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(54) Title: IDENTIFICATION AND CHARACTERIZATION OF INTERACTING MOLECULES BY AUTOMATED INTERACTION MATING

(57) Abstract

The present invention relates to an improved method for the identification and optionally the characterisation of interacting molecules designed to detect positive clones from the rather large numbers of false positive clones isolated by conventional two–hybrid systems. The method of the invention relies on a novel combination of selection steps used to detect clones that express interacting molecules from false positive clones. The present invention provides for high–throughput interaction screens for the reliable identification of interacting molecules, which in turn can lead to the identification of substances inhibiting said interactions. Such inhibitors can find their use in the formulation of a pharmaceutical composition. The present invention further relates to kits useful for carrying out the method of the invention.

IDENTIFICATION AND CHARACTERIZATION OF INTERACTING MOLECULES BY AUTOMATED INTERACTION
MATING

Introduction

The present invention relates to an improved method for the
identification and optionally the characterisation of
interacting molecules designed to detect positive clones from
the rather large numbers of false positive clones isolated by
conventional two-hybrid systems. The method of the invention
relies on a novel combination of selection steps used to
detect clones that express interacting molecules from false
positive clones. The present invention provides for high-
throughput interaction screens for the reliable
identification of interacting molecules, which in turn can
lead to the identification of substances inhibiting said
interactions. Such inhibitors can find their use in the
formulation of a pharmaceutical composition. The present
invention further relates to kits useful for carrying out the
method of the invention.

Protein-protein interactions are essential for nearly all
biological processes like replication, transcription,
secretion, signal transduction and metabolism. Classical
methods for identifying such interactions like co-
immunoprecipitation or cross-linking are not available for
all proteins or may not be sufficiently sensitive. Said
methods further have the disadvantage that only by a great
deal of energy, potentially interacting partners and
corresponding nucleic acid fragments or sequences may be
identified. Usually, this is effected by protein sequencing
or production of antibodies, followed by the screening of an
expression-library.

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An important development for the convenient identification of
protein-protein interactions was the yeast two-hybrid (2H)
system presented by Fields and Song (1989). This genetic
procedure not only allows the rapid demonstration of in vivo
interactions, but also the simple isolation of corresponding
nucleic acid sequences encoding for the interacting partners.
The yeast 2H system makes use of the features of a wide
variety of eukaryotic transcription factors which carry two
separable functional domains: one DNA binding domain as well
as a second domain which activates the RNA-polymerase complex
(activation domain). In the classical 2H system a so-called
"bait" protein comprising of a DNA binding domain (GAL4bd or
lex A) and a protein of interest "X" are expressed as a
fusion protein in yeast ("bait hybrid"). The same yeast cell
also simultaneously expresses a so called "fish" protein
comprising of an activation domain (GAL4ad or VP16) and a
protein "Y" ("fish hybrid"). Upon the interaction of a bait
protein with a fish protein, the DNA binding and activation
domains of the fusion proteins are brought into close
proximity and the resulting protein complex triggers the
expression of the reporter genes, e.g. HIS3 or lacZ. Said
expression can be easily monitored by cultivation of the
yeast cells on selective medium without histidine as well as
upon the activation of the lacZ gene. The genetic sequence
encoding, for example, an unknown fish protein, may easily be
identified by isolating the corresponding plasmid and
subsequent sequence analysis. Meanwhile, a number of variants
of the 2H system have been developed. The most important of
those are the "one hybrid" system for the identification of
DNA-binding proteins, the "tri-hybrid" system for the
identification of RNA-protein-interactions, the "reverse two
hybrid" system, and some systems transferring the 2H approach
to cellular systems other than yeast, namely bacterial and
mammalian (Li and Hershowitz, 1993; SenGupta et al., 1996;
Putz et al., 1996; Vidal et al., 1996; Dove et al., 1997;
Fearon et al., 1992). It should be noted that some 2H systems

do not utilise a transactivation approach. For example, the
functional reconstitution of enzymatic activity.

The classical 2H system for the identification of protein-
protein-interaction, has, until today, only been carried out
on a laboratory scale. Although recent developments have
taken on the challenges in large scale 2H screening (e.g.
Bartel et al.,1996), a successful large scale search of
interacting proteins, for example on the basis of a library
vs. library screen, has not been reported. However, on the
laboratory scale, it is only possible to screen for
interactions between gene products which are known and/or
which are suspected to interact, as the probability of
finding an interaction by random chance is less than 10^{-3}.
The true power of the 2H system, namely finding previously
unsuspected interactions, and even interactions between
previously unknown proteins and protein families, in
screening whole genomes, can only be brought forward in a
large scale approach.

One major difficulty in implementing large scale 2H systems
lies in eliminating the large numbers of false positives not
representing any biologically meaningful interactions between
binding partners. In currently applied 2H systems, in which
proteins of interest, optionally encoded by cDNA libraries,
are fused to a DNA binding domain and an activation domain,
respectively, false positives may arise by several different
mechanisms:
• A peptide or protein cloned into the bait hybrid might
itself have activating properties, activating transcription
of a reporter gene independent of an interaction with the
fish hybrid (herein: "False Positives Class 1").
• A peptide or protein cloned into the fish hybrid might
itself constitute a DNA binding domain, binding to the DNA
binding site or to the basal portion of the promoter,
activating transcription of a reporter gene independent of an

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interaction with the bait hybrid (herein: "False Positives
Class 2").

• A peptide or protein cloned into the fish hybrid might
specifically bind to the DNA binding domain of the bait
hybrid, or, vice versa, a peptide or protein cloned into the
bait hybrid might specifically bind to the activation domain
of the fish hybrid, reconstituting activation of the reporter
gene independent of an interaction between the bait and fish
proteins. This may include binding to epitope tags fused to
the DNA binding domain or activation domain (herein: "False
Positives Class 3").

• Certain peptides or proteins are able to bind non-
specifically to many different other structures (commonly
denoted: "Sticky Proteins"). These will result in a large
number of positives with one common genetic element.

A number of strategies have been previously described which
remove some of the above classes of false positives (Allen et
al., 1995; Bartel et al., 1993).

• The use of two reporter genes (Bartel et al., 1993): One
of these genes usually expresses a selectable marker (e.g.
HIS3) and the other reporter gene a measurable marker
activity (e.g. lacZ), and the reporter gene promoters usually
are different. By scoring positives according to activation
of both reporter genes, this allows removal of a certain part
of the False Positives Class 2 since an interaction with both
of the different promoters is less likely to occur.

• The use of selectable markers and preselection (Bartel et
al., 1996): This method employs replica plating of yeast
clones that express one fusion protein from plates containing
selective medium corresponding to the selectable marker
introduced with the plasmid that encoded said one fusion
protein to plates containing selective medium corresponding
to a reporter gene product (e.g. LEU2 as selectable marker on
plasmid, HIS3 as reporter gene). Yeast clones that showed

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growth on selective medium corresponding to the reporter gene
product where identified as False Positives Class 1 or Class
2, respectively, and were subsequently not used for
interaction mating.

• The use of counterselectable genes and preselection (Vidal
et al., 1996a): Two populations of mating competent yeast
host cells of different mating type are provided that contain
(a) the bait hybrid plasmid and one counterselectable
reporter gene in the population of cells of the first mating
type, and (b) the fish hybrid plasmid and the same or another
counterselectable reporter gene in the population of cells of
the second mating type. When these first and second
populations are kept individually under conditions such that
expression of said counterselectable reporter gene inhibits
the growth of said host cells, False Positives Class 1 and
False Positives Class 2 are hypothetically removed.

• The use of a second, different bait hybrid protein:
Several approaches have been described, all of which are
performed on positive clones after scoring of positives:
(a) curing of the bait hybrid plasmid, transfection with a
second bait hybrid plasmid containing an unrelated bait
protein fused to the same DNA binding domain as in the
original bait hybrid plasmid; expression of the reporter
gene(s) indicates False Positives Class 2 as well as a Sticky
Protein or False Positive Class 3 being fused to the
activation domain (Harper et al., 1993); (b) curing of the
bait hybrid plasmid, transfection with a second bait hybrid
plasmid containing an unrelated bait protein fused to a
different DNA binding domain that binds to a second DNA
binding site controlling a second site comprising the
reporter gene; expression of the reporter gene indicates a
Sticky Protein or certain types of False Positives Class 3
being fused to the activation domain (Le Douarin et al.,
1995); (c) transfection with a control hybrid plasmid
encoding a fusion protein comprising the bait protein and a
second DNA binding domain that binds to a second DNA binding
site controlling a second reporter gene; lack of expression

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of the second reporter gene indicates a False Positive Class
1 (Hurd et al., 1997).

All of these strategies are time and labour consuming, which
is particularly inconvenient in cases where large numbers of
clones are to be analysed, and, in order to eliminate all
false positives, a combination would have to be used,
necessitating even more handling steps. An efficient method
for the elimination of false positives is, however,
inherently more necessary in a library vs. library screen as
compared to the screening of one bait protein against a
library of fish proteins, because the combination of randomly
chosen peptides or proteins/protein fragments with a DNA
binding domain is much more likely to be able to auto-
activate expression of a reporter gene than randomly chosen
peptides or proteins/protein fragments fused to an activation
domain. As a consequence, false positive rates of up to 50 %
would be expected in a library vs. library screen, which,
together with the high total number of clones, does render
such screen unfeasible with conventional 2H methods.

Moreover, as yeast is not the host cell of choice in a
variety of investigations (e.g. when a mammalian protein
suspected to interact with a second protein requires
substantial posttranslational modifications), it would be
desirable for a high throughput 2H system to be versatile
with regard to the type of host cell employed. All systems
put forward so far that are geared to eliminate the
difficulties of 2H screening, although mostly claiming to be
applicable to all types of cells, have been designed towards
the specific biological properties of the yeast two hybrid
system, and cannot necessarily be transferred to, for
example, bacterial or mammalian cell systems.

The technical problem underlying the present invention was
therefore to provide a method that allows the fast and
reliable elimination of false positives. This method should,

moreover, be suitable for large-scale library vs. library
screens using a high-throughput approach. Preferably, this
method would be applicable to a range of different host cell
systems, such as yeast, bacterial, mammalian, plant and
insect cells. Such method could routinely be applied to the
identification of pathways of molecular interactions in
biological systems, and the interconnections between such
pathways. Ultimately, the identification of molecules
involved in interactions that form part of such pathways can
be employed in order to pinpoint targets for pharmaceuticals.

The solution to said technical problem is achieved by
providing the embodiments characterised in the claims.

Detailed description of the invention
Accordingly, in a first aspect the present invention relates
to a method for the identification of at least one member of
a pair or complex of interacting molecules from a pool of
potentially interacting molecules, comprising:

(A)   providing at least one set of host cells, each set
       containing at least one genetic element comprising a
       selectable marker, said selectable marker being
       different between different sets of host cells, said
       genetic elements each comprising genetic information
       specifying one of said potentially interacting
       molecules, said host cells further carrying a readout
       system that is activated upon the presence of auto-
       activating molecules;

(B)   selecting against host cells expressing a molecule able
       to auto-activate the readout system by transferring at
       least one set of host cells or progeny of at least one
       set of host cells to at least one selective medium which
       allows growth of said host cells in the presence of said
       selectable marker different for each set of host cells

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and which precludes growth of said host cells upon auto-
activation of said readout system;

(C)   combining in host cells at least two genetic elements,
       wherein at least one set of host cells grows on said
       selective medium specified in (B);

(D)   allowing at least one interaction, if any, to occur;

(E)   selecting for said interaction by transferring said host
       cells or progeny of said host cells to a selective
       medium that allows identification of said host cells
       upon activation of the readout system;

(F)   identifying host cells that contain interacting
       molecules that activate said readout system on said
       selective medium;

(G)   identifying at least one member of said pair or complex
       of interacting molecules;

wherein said host cells are not yeast cells.

Furthermore, in a second aspect the present invention relates
to a method for the identification of at least one member of
a pair or complex of interacting molecules from a pool of
potentially interacting molecules, comprising:

(A)   providing at least one set of host cells, each set
       containing at least one genetic element comprising a
       selectable marker, said selectable marker being
       different between different sets of host cells, said
       genetic elements each comprising genetic information
       specifying one of said potentially interacting
       molecules, said host cells further carrying a readout
       system that is activated upon the presence of auto-
       activating molecules;

(B)   selecting against host cells expressing a molecule able
        to auto-activate the readout system by transferring at
        least one set of host cells or progeny of at least one
        set of host cells to at least one selective medium which
        allows growth of said host cells in the presence of said
        selectable marker different for each set of host cells
        and visual differentiation between those cells whose
        readout system has been activated from those host cells
        whose readout system has not been activated;

(C)   combining in host cells at least two different genetic
        elements, wherein at least one set of host cells grows
        on said selective medium specified in (B);

(D)   allowing at least one interaction, if any, to occur;

(E)   selecting for said interaction by transferring said host
        cells or progeny of said host cells to a selective
        medium that allows identification of said host cells
        upon activation of the readout system;

(F)   identifying host cells that contain interacting
        molecules that activate said readout system on said
        selective medium;

(G)   identifying at least one member of said pair or complex
        of interacting molecules

Furthermore, in a third aspect the invention relates to a
method for the identification of at least one member of a
pair or complex of interacting molecules from a pool of
potentially interacting molecules, comprising:

(A)   providing at least one set of host cells, each set
        containing at least one genetic element comprising a
        selectable marker, said selectable marker being

different between different sets of host cell, said
genetic elements each comprising genetic information
specifying one of said potentially interacting
molecules, said host cells further carrying a readout
system that is activated upon the presence of auto-
activating molecules;

(B)   selecting against host cells expressing a molecule able
       to auto-activate the readout system by transferring at
       least one set of host cells or progeny of at least one
       set of host cells to at least one selective medium which
       allows growth of said host cells in the presence of said
       selectable marker different for each set of host cells
       and which precludes growth of said host cells upon auto-
       activation of said readout system;

(C)   combining in host cells at least two genetic elements,
       wherein at least one set of host cells grows on said
       selective medium specified in (B);

(D)   allowing at least one interaction, if any, to occur;

(E)   selecting for said interaction by transferring said host
       cells or progeny of said host cells to a selective
       medium that allows identification of said host cells
       upon activation of the readout system;

(F)   identifying host cells that contain interacting
       molecules that activate said readout system on said
       selective medium;

(G)   identifying at least one member of said pair or complex
       of interacting molecules;

wherein said host cells are yeast cells, and at least one of
the steps (B), (C), (E) or (F) is effected or assisted by
automation using regular grid patterns of host cells.

It shall be understood herein, that where referral is taken to the "method of the invention" or the "invention", respectively, this shall refer to all three aspects of the invention as described above, whereas when referral is taken to a specific aspect of the invention, namely the first second and third aspect, this shall refer to only this aspect of the invention.

The terms "identification" and "identifying", as used in accordance with the present invention, relate to the ability of the person skilled in the art to detect positive clones that express interacting molecules from false positive clones due to the activation of the readout system on the selective media and optionally additionally to characterise at least one of said interacting molecules by one or a set of unambiguous features. Preferably, said molecules are characterised by the DNA sequence encoding them, upon nucleic acid hybridisation or isolation and sequencing of the respective DNA molecules. Alternatively and less preferred, said molecules may be characterised by different features such as molecular weight, isoelectric point and, in the case of proteins, the N-terminal amino acid sequence etc. Methods for determining such parameters are well known in the art.

The term "potentially interacting molecule(s)", as used in accordance with the present invention, relates to nucleic acids, peptides, domains of proteins or proteins that can be formed upon the transcription and/or translation of genetic information, and which may but are not required to be able to interact with one or more other such nucleic acids, peptides or proteins, together forming a pair or complex of interacting molecules. Preferably, said potentially interacting molecules represent nucleic acids, peptides, domains of proteins or proteins which occur in cells from which the genetic information was derived.

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Preferably, said potentially interacting molecules specified by said genetic information are connected to a further entity that will upon the interaction activate or contribute to the activation of said read out system. It is further preferred that said entity is conserved for each type of genetic element and that different types of genetic elements comprise different entities. It is additionally preferred that said potentially interacting molecule forms, when transcribed as RNA from said genetic element, an RNA transcript fused with RNA specifying said entity. Most preferably, said fused RNA transcript is translated to form a fusion protein comprising said potentially interacting molecule fused to said entity. As will be elaborated further herein below, said entity may be in one type of genetic element a DNA sequence encoding a DNA-binding domain and in a different type of genetic element a transactivating protein domain. Preferably, said genetic elements are vectors such as plasmids. The at least two genetic elements comprised in said host cell preferentially contain genetic information from a library such as a cDNA or genomic library. Thus, the method of the invention allows the screening of a variety of host cells wherein the vector portion of said genetic elements is preferably the same for each type of genetic element whereas the potentially interacting molecules are representatives of a library and, thus, as a rule and in case that the library has not been amplified, may differ in each host cell or in a majority of host cells. In this connection the term "type of genetic element" refers to an element characterised by comprising the same entity, selectable and, optionally, counterselectable markers.

The genetic elements specified in the present invention may further and advantageously be equipped with selection markers functional in bacteria such as E.coli. The selection markers, for example aphA (Pansegrau et al., 1987) or bla allow the easy separation of said genetic elements upon retransformation into E.coli strains.

Preferably, the interaction according to the invention is a specific interaction. Preferably, the "interaction" of said molecules is characterised by a high binding constant. However, the term "interaction" may also refer to a binding between molecules with a lower binding constant which, however, must be sufficient to activate the readout system. The interaction that is detectable by the method of the invention preferably leads to the formation of a functional entity having a biological, physical or chemical activity which was not present in said host cell before said interaction occurred. More preferably, such activity is a detectable activity. Most preferably, such functional entity is a protein.

Said interaction may preferably lead to the formation of a functional transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a responsive moiety driving the activation of said readout system. For example, said moiety may be a promoter. Alternatively for example, said interaction may lead to a detectable fluorescence resonance energy transfer obtained by the interaction of fusion proteins containing, for example, the GFP type a and GFP type b fluorescent proteins (Cubbitt et al., 1995).

The term "growth on selective media" refers to the fact that yeast cells containing one genetic element are placed on selective media that precludes growth of said cells upon auto-activation of said readout system, or the visual differentiation between cells whose readout system has been auto-activated and those cells whose readout system has not been auto-activated. For example, when a ura3 yeast strain which contains a URA3 reporter system and which also contains a plasmid expressing a LexA fusion protein that activates the URA3 reporter system is selected on selective medium containing 5-fluoroorotic acid (5-FOA), the yeast cells

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cannot grow on this medium because the URA3 reporter system
synthesises the enzyme orotidine-5'-phosphate decarboxylase
that converts 5-FOA into the toxic compound 5-fluorouracil
(Boeke et al., 1984). In contrast, on a selective medium
lacking for example, tryptophan and which contains X-Gal,
yeast cells that contain plasmids for the expression of LexA
fusion proteins that either activate or do not activate the
readout system can grow. However, the yeast cells in which
the lacZ reporter system is activated will turn blue because
the substrate X-Gal is cleaved into the coloured compound 5-
bromo-4-chloro-indigo.

The term "growth on selective medium" also refers to the fact
that host cells containing two genetic elements expressing
interacting molecules which do not activate the readout
system on their own, are selected on selective medium. For
example, clones that express interacting LexA and GAL4ad
fusion proteins which activate a URA3 and HIS3 reporter
system can be selected on selective media lacking tryptophan,
leucine histidine and uracil. On this selective medium, only
those yeast cells that contain interacting LexA and GAL4ad
fusion proteins that activate the URA3 and HIS3 readout
system can grow.

When in accordance with the present invention host cells are
selected on at least one selective medium precluding growth
in the presence of a counterselectable marker, it should be
noted that each of the selective media would comprise at
least one counterselectable compound such as 5-FOA or
cycloheximide wherein the counterselectable compound would be
different in different selective media; they would further
typically lack a compound complementing for an auxotrophic
marker or comprise an antibiotic. The compound or antibiotic
may be the same for the various selective media. Preferably,
at least one is different.

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In accordance with the present invention it is envisaged that a counter selection against clones that express a single molecule able to activate the URA3 readout system can be carried out on culture media preferably comprising 5 fluoroorotic acid (5-FOA). By applying this selection step prior to the preferably automated interaction mating, those clones that express auto-activating fusion proteins can be eliminated from a library of clones.

The terms "auto-activate" or "auto-activation" relate to the fact that certain molecules encoded by said genetic elements are able to activate the readout system without the need for an interacting molecule. For example, the single fusion protein LexA-HIP1 is capable of activating the HIS3 and lacZ readout system without any corresponding interacting activation domain fusion protein (Wanker et al., 1997).

The term "preselection", as used in accordance with the present invention, relates to the selection of a set of host cells comprising a genetic element and a readout system for those host cells expressing a single fusion protein unable to auto-activate said readout system. The term "genetic preselection", as used in accordance with the present invention, relates to preselection emplying counterselection which makes use of a readout system comprising a counterselectable gene.

The present invention provides a highly effective method to perform 2H screens in a variety of host cell types. The invention provides a reliable method for the detection of false positive clones that express fusion proteins which are able to activate the readout system without an interaction with a second molecule. Further aspects provide methods to produce pharmaceutical compositions employing large scale 2H methods. Finally, kits are provided that will allow performing the method of the invention.

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In applying 2H techniques to the screening for interacting
molecules, it is highly desirable to remove as many False
Positives Class 1 and Class 2 as referred to above before
allowing an interaction, if any, to occur, in order to reduce
the total number of false positives that need to be handled
in further steps. In the above aspects of the invention, the
present invention provides for three methods to achieve such
removal of false positives employing preselection. Inclusion
of at least one preselection step as a feature of the
invention has a number of significant advantages as compared
to prior art methods that we addressed in more detail below.

In accordance with the first aspect of the present invention,
said removal of false positives is achieved by
counterselection of host cells comprising a counterselectable
reporter gene wherein said host cells are not yeast cells.

Although genetic preselection has been shown in yeast 2H
systems, so far this concept has not been applied to other
cellular systems. While counterselectable reporter genes are
readily available for, for example, bacterial systems, and
although bacterial 2H systems were available (Dove et al.,
1997), the use of genetic preselection in bacterial system
has not been described explicitly. This is surprising, as
bacterial systems are well suited for certain applications in
protein-protein interaction screening as is further expanded
on below, and genetic preselection can be advantageously
applied in large scale 2H screening. For example, Vidal et
al. (1996a), although claiming that the system described is
applicable to a wide range of cells, no attempt is made to
teach how to overcome the specific challenges the
implementation of genetic preselection in bacterial 2H
screening would pose.

In the second of the aforementioned aspects of the invention,
a preselection step for host cells expressing a single
molecule unable to activate the readout system employs visual

differentiation between host cells whose readout system has
been activated and host cells whose readout system has not
been activated. In a preferred embodiment of this aspect of
the invention, the readout system comprises at least one
detectable protein. More preferably, said detectable protein
is encoded from at least one of the genes lacZ, gfp, yfp,
bfp, cat, luxAB, HPRT or a surface marker gene. Other such
genes exist and the person skilled in the art will readily
identify other such genes that can be employed according to
this embodiment.

With respect to this second aspect of the invention, it is
additionally preferred, that said visual differentiation in
step (B) is based on a difference between host cells in
different activation states of the readout system which can
be detected by visual means. It is particularly preferred
that said difference between host cells in different
activation states that can be detected by visual means is
brought about by activation of one of the genes lacZ, gfp,
yfp, bfp, CAT, luxAB, or of a surface marker.

Most preferably, said visual means include digital image
capture, storage, processing and/or analysis.

Such visual means may incorporate a camera, a sensitive CCD
camera that is suitable for luminescent and fluorescent
detection, or may be colourimetric detection systems
including computer-based scanners or specialised fluorescent,
luminescent or colourimetric plate readers such as the Victor
II system from Wallace (Finland).

Preselection employing one or more counterselective reporter
genes or, alternatively, by visually detecting host cell
expressing a single fusion protein able to activate the
readout system, can equally be used to remove false positive
clones in the 2H system. Using a counterselective reporter
gene, however, is in some cases unsatisfactory for a number

of reasons, particularly when applied to a large-scale
library vs. library screen with the aim of generating protein
interaction networks of a eukaryotic system. First, it is
known that during counterselection using media containing
counterselective compounds such as 5-FOA, many yeast cells
that express the counterselective marker may not be killed,
but rather remain dormant and become viable when transferred
to a medium free from a counterselective compound. This
effect can lead to a `leaky' genetic preselection system
which may lead to a significant number of false-positive
colonies being found in an interaction library. This is
particularly so when a library vs. library screen is
conducted, as even a small number of, e.g. False Positives
Class 1, each of which will activate the readout system
regardless of its partner protein, will make the task of
finding a small number of true positives next to impossible.
Second, because many yeast colonies from a library of cells
are of different sizes, each containing a different number of
cells, collecting surviving cells by scraping or washing off
colonies from a counterselective plate will skew the
representation of particular inserts from a cloned and plated
library. Third, for many host-cell types including mammalian
systems, counterselective genes are not available or are
difficult to enable. Finally, the sensitivity of a
counterselective approach is low since fusion proteins that
are weak auto-activators of the readout system will cause
insufficient reporter gene transcription to cause cell death
through counterselection. In contrast, the readout system
commonly used to finally assay any protein-protein
interaction between two fusion proteins in the 2H system is
the significantly more sensitive ß-gal assay. Therefore, many
single fusion proteins able to auto-activate the
counterselective readout system but not sufficiently to cause
cell death would cause a detectable signal from the more
sensitive ß-gal readout system at a later step.

By preselecting against false positive clones using the same
readout system as is used to assay for potential interaction
between two fusion proteins at later steps in a 2H screen,
the amount of false-positive clones passing through the
preselection step can potentially be reduced. Furthermore, by
allowing all clones that carry a plasmid to grow and using
visual differentiation to distinguish false positive clones,
false positive clones could be ignored using an automated
colony picking system. This would significantly reduce the
problem of false positive clones being carried through the
preselective step compared to a counterselective system that
is `leaky' since the location of dormant yet viable cells is
unknown. Also, it is well known in the art that readout
systems exhibiting visual differentiation between activation
and non-activation states, such as ß-galactosidase, green
fluorescent protein, luciferase, secreted alkaline
phosphatase and ß-glucuronidase, are detectable when
expressed in different host-cell types including yeast,
bacteria, plant and insect cells. Therefore, systems to
preselect for false positive clones would be easier to
transfer to other host-cell types if based on these readout
systems.

In the third aspect of the present invention, a method is
disclosed subjecting yeast host cells to genetic preselection
in a yeast 2H screen, wherein at least one of the steps (A),
(C), (E) or (F) is effected or assisted by automation using
regular grid patterns of host cells. The cumbersome and
highly repetitive nature of the experimental steps involved
in large scale 2H experiments makes automation of these steps
seem an obvious choice. However, although several authors
have previously indicated introducing automation to 2H
techniques, it has so far not been shown how a high
throughput, automated 2H screen could be performed. In the
field of molecular biology, there are a host of different
ways by which automation may be conducted, e.g. by using
pipetting robots, plate readers, automated sequencing

machines etc., but most of these have been developed with the
aim to automate the handling of large numbers of different
molecules rather than large numbers of different cells or
clones. A person skilled in the art could therefore not
conclude how to perform high throughput 2H screening from the
simple proposal to include automation. Vidal et al. (1996) as
well as Hurd et al. (1997) merely mention the possibility of
automating the 2H systems they propose without substantiating
how to implement this feat; Nandabalan et al., (1997) purport
to have introduced automation to 2H screens, enabling large
throughput, yet the system they have devised represents
exclusively high throughput identification of nucleic acid
sequences encoding interacting proteins after clones have
been manually handled until identification of positives. The
key to automation herein lies in the use of regular grid
patterns together with suitable devices and procedures, which
automatically process said regular grid patterns.

In a preferred embodiment of the method of the present
invention said pair or complex of interacting molecules is
selected from the group consisting of RNA-RNA, RNA-DNA, RNA-
protein, DNA-DNA, DNA-protein, protein-protein, protein-
peptide, or peptide-peptide interactions.

Accordingly, the method of the invention is applicable in a
wide range of biological interactions. For example, the
invention will be useful in identifying peptide-protein or
peptide-peptide-interactions by employing synthetic peptide
libraries (Yang et al., 1995).

Two applications of interest are the application of a large
scale 2H system for the detection of protein-protein
interactions involved in medically relevant pathways which
may be useful as diagnostic or therapeutic targets for the
treatment of disease, and a large scale tri-hybrid system
which is one example of said complex of interacting molecules
mentioned herein above for the identification of, for

example, novel post-transciptional regulators and their
binding sites (SenGupta et al., 1996; Putz et al., 1996). In
this regard it should be noted that a complex, in accordance
with the invention may comprise more than three interacting
molecules. Furthermore, such a complex may be composed of
biologically or chemically different members. For example, to
identify interacting RNA binding proteins and RNA molecules,
a plasmid expressing a LexA-HIV-1Rev protein, a plasmid
transcribing an RNA sequence in fusion with the responsive
element and a plasmid expressing a potentially RNA-
interacting protein in fusion with an activation domain may
be present in one cell. The plasmids encoding the RNA fusion
molecule and the activation domain fusion protein must
contain different selectable and counterselectable markers
according to the method of the invention. If the RNA fusion
molecule interacts with the respective two fusion proteins,
the readout system is activated. To test whether the RNA
fusion molecule or the activation domain fusion protein
interact, the method of the invention is used to investigate
the activation of the readout system in the absence of either
of these fusion molecules.

In a further preferred embodiment, said genetic elements are
plasmids, artificial chromosomes, viruses or other
extrachromosomal elements.

Whereas it is preferred, due to the easy handling, to employ
plasmids that specify the genetic elements in accordance with
the present invention, the person skilled in the art will be
able to devise other systems that carry said genetic
elements. Furthermore, the person skilled in the art will be
well aware that the preferred genetic element will depend on
the host cell system. For example, retroviral vectors might
be employed in mammalian host cells.

In another preferred embodiment, said readout system
additionally comprises at least one counterselectable gene.

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As the biological principle of counterselection is well known in the art, the person skilled in the art may choose from a variety of such counterselectable genes. Preferably, said genes are URA3, LYS2, sacB, CAN1, CYH2, rpsL, or lacY. The person skilled in the art will be able to choose the appropriate marker for a given cell system, e.g. URA3 in a yeast 2H system or sacB in a bacterial system.

Preferably, said selective medium in step (B) of the method of the invention comprises a counterselective compound. More preferably, said counterselective compound is chosen from 5-FOA, canavanine, cycloheximide, sucrose, streptomycin or.tONPG.

In this embodiment, for example, the URA3 gene is incorporated as a component of the readout system. Clones containing only one of said genetic elements are placed on a selective medium comprising 5-FOA. In the case that clones that express a single molecule able to activate the readout system, 5-FOA is converted into the toxic 5-fluorouracil. Accordingly, host cells containing auto-activating molecules will die on the selective medium containing 5-FOA (Le Douarin, 1995, Vidal et al., 1996a). Surviving cells are then collected by scraping or washing off colonies from the surface.

In an additional preferred embodiment, the readout system according to the invention comprises at least one detectable protein. A number of readout systems are known in the art and may, if necessary, be adapted to be useful in the method of the invention.

Most preferably, said detectable protein is that encoded by the genes lacZ, HIS3, URA3, LYS2, sacB, tetA, gfp, yfp, bfp, CAT, luxAB, HPRT or a surface marker, respectively. As is well known in the art, the expression of the ß-gal enzyme in

yeast can be used for the formation of a detectable blue
colony after incubation in X-Gal solution. Proteins which
confer resistance to an antibiotic represent a popular choice
for bacterial cell systems and can be detected by selection
for growth in the presence of the antibiotic. Expression of
fluorescent proteins, as well as the expression of a surface
marker and subsequent visualisation with a fluorescently
marked antibody, can preferentially be employed in mammalian
systems in conjunction with fluorescence assisted cell
sorting (FACS) or laser scanning confocal microscopy. Of
course, the method of the invention is not restricted for use
of only one readout system. On the contrary, if desired, a
number of such readout systems may be combined. Said
combination of a number of readout systems is, in accordance
with the present invention, also comprised by the term
"readout system". Such a combination will provide an
additional safe guard for the identification of clones
containing interacting partners.

Although the 2H system has been developed in yeast, the
method of the invention can be carried out in a variety of
host systems. Preferred of those are yeast cells, bacterial
cells, mammalian cells (Wu et al. 1996), insect cells, plant
cells or hybrid cells. Preferably, the bacterial cells are E.
coli cells.

It is understood in the art that to identify, detect or assay
the variety of different protein-protein interactions that
exist in biological systems, it is likely that a variety of
host systems will have to be employed. For example,
prokaryotic systems have certain advantages over eukaryotic
systems including the ease of genetic, laboratory and
automated procedures. Additionally, unlike conventional yeast
two-hybrid systems, nuclear localisation of fusion proteins
is irrelevant for prokaryotic cells and the entry of small
molecules into the cell is typically easier than for a yeast
cell. However, some protein-protein interactions depend on

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post-translational modifications such as mRNA splicing or
glycosylation that are not available in prokaryotic or yeast
cells, respectively. Therefore, in order to uncover many, if
not most, protein-protein interactions that exist in
biological systems, library vs. library interactions screens
will need to be conducted in a variety of host types. The art
would benefit from an improved two-hybrid system that can
deal with the large numbers of clones and false-positive
clones generated while conducting these screens in a variety
of host-types. It would be of great advantage if such a
system were available that functioned or was conducted in a
substantially similar manner regardless of the host-cell type
used. Although other methods to conduct large-scale two-
hybrid screens claim to be applicable to all types of cells,
they are typically geared towards only one cell type, in most
cases yeast. For example, Vidal et al (1996a) describes a
genetic method to preselect against cells expressing single
fusion proteins able to activate the readout system, but no
solution is provided as to how a person skilled in the art
may conduct this preselection in for example a prokaryotic or
mammalian two-hybrid system. Using detectable proteins such
as GFP or ß-galactosidase that are appropriate for a broad
range of host-types as one part of the readout system, a
substantially similar procedure and method can be used to
visually differentiate against false positive clones in a
variety of host-types.

Of course, the genetic elements may be engineered and
prepared in one host organism and then, e.g. by employing
shuttle vectors, be transferred to a different host organism
where it is employed in the method of the invention.

Whereas the person skilled in the art may initiate the
identification method of the invention starting from fully
transformed or transfected host cells, he may wish to first
generate such host cells in accordance with the aim of his
research or commercial interest. For example, he may wish to

generate a certain type of library first that he intends to
screen against a second library already present in said host
cells. Alternatively, he may have in mind to generate two or
more different libraries that he wants to screen against each
other. In this case, he would need to first transform said
host cells, simultaneously or successively, with both or all
types of genetic elements.

In another preferred embodiment, the method of the present
invention comprises transforming, infecting or transfecting
at least one set of host cells of said sets of host cells
with said genetic element or genetic elements prior to step
(D).

In another preferred embodiment, the method of the invention
further comprises transforming, infecting or transfecting
each set of host cells of said sets of host cells with dais
genetic elements prior to step (D).

In another preferred embodiment, the method of the present
invention comprises transforming, infecting or transfecting
one set of host cells of said sets of host cells with at
least one genetic element prior to step (A), selecting
against host cells in said one set of host cells expressing a
molecule able to auto-activate said readout system as
specified in step (B), and transforming, infecting or
transfecting said set of host cells with at least one further
genetic element prior to step (D).

In another preferred embodiment, said host cells with said
genetic elements are generated by cell fusion, conjugation or
interaction mating prior to step (D), preferable in step (C).

In a particularly preferred embodiment, said cell fusion,
conjugation or interaction mating is affected or assisted by
automation. More preferably, said automation is effected by
an automated picking, spotting, rearraying, pipetting,

micropipetting or cell sorting device. Most preferably, said
device is a picking robot, spotting robot, rearraying robot,
pipetting system, micropipetting system or fluorescence
assisted cell sorting (FACS) system.

Interaction mating is well known as a tool for use in the
yeast 2H system to combine genetic elements that express
potentially interacting fusion proteins (Bendixen et al.,
1994). Although cell fusion, conjugation or interaction
mating are efficient in combining genetic material between
different cell strains, such an approach would only be of use
in a large-scale library vs. library screen if it could be
conducted at high-throughput, due to the large number of
colonies that needs to be harvested. By utilising automated
systems which had been designed to speed the handling of
E.coli cells for the analysis of DNA (Lehrach et al, 1997),
it is possible to conduct automated and high-throughput
interaction mating in bacteria and yeast cells. Pipetting or
micropipetting systems could be used for example in the
handling of mammalian cells. Alternatively, FACS could be
employed to the same task.

Although picking of E.coli clones for DNA analysis using
vision-controlled robotic systems such as described in
Lehrach et al. (1997) is well known, the large-scale robotic
picking of yeast clones was not considered by the skilled
person because of the difficulties of dealing with this
organism. For example, yeast colonies typically have variable
size, shape and colour when growing on solid agar and often
grow on an opaque lawn of non-transformants obstructing
visual colony recognition. Secondly, a large-amount of cell
material is needed to successfully inoculate further cultures
compared to E.coli, and finally, ethanol alone cannot be
reliably used to sterilise picking tools between picking
cycles.

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However, for the reliable picking of clones from, for example, a yeast 2H screen, suitable changes to a standard picking robot as described by Lehrach et al. (1997) had to be devised.

First, the illumination of the agar-trays containing plated colonies was changed from the dark-field sub-illumination typically used when picking E.coli clones to dark-field top-illumination to successfully visualise yeast colonies by differentiation from the lawn of non-transformant cells. The existing vision guided motion system (Krishnaswamy & Agapakis 1997) was modified to allow for a larger range of `blob' size when selecting yeast colonies to pick from the blobs features returned by connectivity algorithms when applied to a digital image of the agar tray containing colonies. Secondly, the clone inoculation routine was re-programmed to ensure that cell material which had dried on the picking pins during the picking routine was initially re-hydrated by 10 seconds of immersion in the wells of a microtiter plate before vigorous pin-motion with the well. This robotic procedure ensured that sufficient cell material was inoculated from each picking pin into an individual well of a microtiter plate. Finally, the picking pins were sterilised after inoculation to allow the picking cycle to be repeated by programming the robot to brush the picking pins in a 0.3% (v/v) solution of hydrogen peroxide, followed by a 70% ethanol rinse from a second wash-bath and finally a heat-gun treatment to evaporate any remaining ethanol from the pins.

The combination at high-throughput of genetic material from all combinations of pairs of cells expressing fusion proteins could also be conducted in a systematic, rather than a random manner. To minimise the number of false positive entering such an automated combination strategy to identify interacting molecules, it would be preferable to conduct the combination with libraries of cells from which false positive

cells had been removed or minimised by genetic preselection or visual differentiation as referred to above.

It will be clear to a person skilled in the art that the approach described here will be able to create regular grid patterns of densities greater than 2 to 10, 10 to 100, 100 to 500 or 500 to 1000 clones per square centimetre, depending on the automated system and host cell type used. By way of illustration, these may be created by using a robotic pipetting or piezo dispensing system carrying one clone to a specific location containing another clone, or by using said approaches to contact cells of one mating type to a lawn of at least one clone of another mating type. Said lawn may be applied as a layer of cells suspended in a solid or semi-solid growth medium or may be applied by spraying a thin and uniform layer of cells of one mating type onto the surface where contact with the cell of the alternative mating type is made. Of particular advantage are systems where individual clones can be individually positioned or contacted with other particular clones. This can be enabled for example by individually addressable multi-head dispensing units, or by a transfer head with individually addressable and moveable transfer pins. Such a system can easily be brought forward by a person skilled in the art using the disclosures in this invention using systems such as the rearraying robots as described by Stanton et al (1995) and Lehrach et al (1997), or from those supplied by commercial robot suppliers such as Genetix (UK). It should be recognised that said combination strategy may be conducted on a planar carrier as disclosed herein below. It may also be conducted directly on solid growth agar, or within the wells of microtiter plates.

It may be that for some library vs. library interaction screens, the number of positive clones obtained by making all possible combinations of interaction mating is low. For a systematic clone vs. clone interaction screen of two libraries each of 10,000 fusion proteins, a minimum of 5 x

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10^7 combinations need to be tested. If it is assumed that any given fusion protein will have approximately 10 possible interaction partners, only around 10^4 positive clones and hence protein-protein interactions will be detected from such a screen. Because the efficiency of interaction mating is so high (Sherman et al, 1984), in these cases it would be possible to conduct such large-scale interaction screens more efficiently by contacting individual cells from the different libraries using pools of different clones. Clones from a given library would be pooled in numbers of 2 to 10, 10 to 100, 100 to 500 or 500 to 1000, and pools contacted with clones or pools from a second library. Preferable, said pools of clones shall be designed using multidimensional pooling strategies as are commonly known in the art (Barillot et al, 1991; Strauss, et al, 1992; Liu et al. 1995) such that the individual identity of the two clones that contacted and caused activation of the readout system can be subsequently deconvoluted. It is of advantage that most or all false positive clones are removed from the two libraries prior to combination such that said deconvolution can be conducted most efficiently.

It is further preferred in accordance with the present invention that the selectable markers are auxotrophic or antibiotic markers.

It is important to note that some of the markers that are used as a readout system, may also be used as selectable markers. It is further important to note that one and the same marker can not be used as selectable marker and as part of the readout system at the same time.

Most preferably, said auxotrophic or antibiotic markers are selected from LEU2, TRP1, URA3, ADE2, HIS3, LYS2, kan, bla, Zeocin, neomycin, hygromycin, pyromycin or G418.

Planning of experiments may require that the test for
interaction need not be done immediately after the provision
of host cells and, possibly, the occurrence of the
interactions. In such cases, it may be desirable to store the
transformed host cells for further use. Accordingly, a
further preferred embodiment of the invention relates to a
method wherein progeny of host cells obtained in step (C) are
transferred to a storage compartment.

In particular in cases where a large number of clones is to
be analysed, said transfer to a storage compartment is
advantageously effected or assisted by automation. More
preferably, said automation is effected by an automated
arraying, picking, spotting, pipetting, micropipetting or
cell sorting device. Most preferably, said device is an
arraying robot, picking robot, spotting robot, automated
pipetting or micropipetting system or FACS system. For
example, a pipetting, micropipetting or FACS system may be
advantageously applied to the transfer of mammalian cells.
Other automation or robot systems that reliably transfer
progeny of said host cells into predetermined arrays in the
storage compartments may also be employed. As the person
skilled in the art will realise, the choice of said device
will largely depend on the host cell system under
investigation.

The host cells may, in this embodiment, be propagated in said
storage compartment and provide further progeny for the
additional tests. Preferably, replicas of said storage
compartment maintaining the array of clones are set up. Said
storage compartments comprising the transformed host cells
and the appropriate media may be maintained in accordance
with conventional cultivation protocols. Alternatively, said
storage compartments may comprise an anti-freeze agent and
therefore be appropriate for storage in a deep-freezer. This
embodiment is particularly useful when the evaluation of
potential interacting partners is to be postponed. As is well

known in the art, frozen host cells may easily be recovered
upon thawing and further tested in accordance with the
invention. Most preferably, said anti-freeze agent is
glycerol which is preferably present in said media in an
amount of 3 - 25% (vol/vol), or DMSO.

In a further particularly preferred embodiment of the method
of the invention, said storage compartment is at least one
microtiter plate. Most preferably, said at least one
microtiter plate comprises 96, 384, 846 or 1536 wells.
Microtiter plates have the particular advantage of providing
a pre-fixed array that allows the easy replicating of clones
and furthermore the unambiguous identification and assignment
of clones throughout the various steps of the experiment.
384, 846 or 1536 well microtiter plates are, due to
comparatively small size and large number of compartments,
particularly suitable for experiments where large numbers of
clones need to be screened, but plates with lower numbers of
cells may be required depending on the host cell system.

Depending on the design of the experiment, the host cells may
be grown in the storage compartment such as the above
microtiter plate to logarithmic or stationary phase. Growth
conditions may be established by the person skilled in the
art according to conventional procedures. Cell growth is
usually performed between 15 and 45 degrees Celsius.

Referring to step (E), in a preferred embodiment of the
method of the invention, the transfer of said host cells or
progeny of host cells is effected or assisted by automation
using a regular grid pattern. More preferably, said
automation using a regular grid pattern transfer of said host
cells or progeny of host cells is effected by an automated
picking, spotting, replicating, pipetting or micropipetting
device. Most preferably that device is a picking robot,
replicating robot, spotting robot, pipetting system,
micropipetting system or fluorescent assisted cell sorting

(FACS) system. How such a robot or automated system may be devised and equipped is, for example, described in Lehrach et al. (1997). Other automation or robot systems that reliably transfer progeny of said host cells into predetermined arrays in the storage compartments may also be employed. By using a computer-controlled pipetting system according to the invention, regular grid patterns of high density could be created. According to this embodiment of the invention, planar carriers with a high-density pattern of yeast clones from the defined interaction library contained within 384-well microtiter plates are provided by using a high-throughput spotting robot such as that described by Lehrach et al. (1997). Further, a regular grid pattern of yeast cells expressing fusion proteins at a density greater than 18 clones per square centimetre within 1536-well microtiter plates, which have a well every 2.25 mm in a 32 by 48 well arrangement, a regular grid, is provided. As the person skilled in the art will realise, the choice of said device will largely depend on the host cell system under investigation.

In order to increase the population of host cells available for growth on said selective medium in step (E), it is most advantageous to make multiple transfers that carry additional host cells of the same clone to the same position in said regular grid. Preferably, the number of said multiple transfers is between 2 and 20 times. If said multiple transfer is from a microtiter plate and effected or assisted by automation it is most advantageous to be made from a slightly different position of the microtiter plate well containing said clone.

In a preferred embodiment of the invention, said transfer is made to at least one carrier.

In another preferred embodiment, this at least one carrier is a microtiter plate, and the regular grid pattern is at

densities greater than 1, preferably greater than 4, more
preferably greater than 10, most preferably greater than 18
clones per centimeter square.

In yet another preferred embodiment, said at least one
carrier is a porous support and the regular grid pattern is
at densities in the range of 1 to 10, preferably 10 to 50,
more preferably 50 to 100, most preferably greater than 100
clones per centimeter square.

In yet another preferred embodiment, said at least one
carrier is a non-porous support and the regular grid pattern
is at densities in the range of 1 to 100, preferably 100 to
500, more preferably 500 to 1000, most preferably greater
than 1000 clones per centimeter square.

The progeny of said host cells may be transferred to a
variety of carriers. It is well known in the art that many
enzymatic screens can be conducted at high throughput in
microtiter plates. Microtiter plates are robotically handled,
filled, incubated and any signal from the enzymatic screen
measured. Indeed, this approach forms the basis of most high-
throughout screen in the pharmaceutical industry to identify
primary hits from large chemical libraries. Each well in such
a screen contains identical cells or other biological system,
and it is only the small amount of test chemical that differs
in each well of the microtiter plate. In contrast, a library
of host-cells expressing fusion proteins effectively
comprises a different biological system in every well (host-
cell expressing two potentially interacting fusion proteins)
that must be screened for activity of the readout system. If
a screen to identify interacting positive cells that express
interacting molecules could be conducted using microtiter
plates, then it would be possible to use substantially
similar robotic systems to that currently developed for high-
throughput enzymatic screens.

A person skilled in the art will recognise, that although the
Yeast One Step Yeast Lysis Buffer supplied by Tropix (USA) is
a convenient method to lyse cells for a microtiter plate
format screen, other methods are appropriate. Other methods
to lyse host cells are well known in the art and include
lysis of cells stored in a microtiter plate without anti-
freeze medium by a freeze-thaw procedure, or by addition of a
small amount of toluene/chloroform mixture. Other ß-
galactosidase substrates equally may be used including X-Gal,
and the activity of the reporter gene measured by
colourimetric means from the density of the blue-colour
produced. Indeed, other readout systems may be utilised that
do not depend on cell lysis. For example, secreted enzymes
such as secreted alkaline phosphatase, or cell-surface or
secreted proteins that may be detected by ELISA assay.
Readout systems that do not depend on additional substrates,
for example green fluorescent protein, may also be utilised.
The method of detection used will depend on the readout
system used, and may include a sensitive CCD camera that is
suitable for luminescent and fluorescent detection, or may be
colourimetric detection systems including computer-based
scanners or specialised fluorescent, luminescent or
colourimetric plate readers such as the Victor II system from
Wallace (Finland). A person skilled in the art would also be
able to design a readout system based on radioactive
detection using for example a scintillation counter or
phosphor storage imaging (Johnston et al., 1990).

This carrier might also be a porous support, e.g. a membrane
manufactured from nylon, nitro-cellulose, cellulose acetate
or PVDF, which membrane would be particularly advantageous
for bacterial cells or yeast cells. Said solid support could,
for example be a glass slide coated with poly-lysine, which
glass slide would be particularly advantageous for mammalian
cells. Solid supports can be advantageous, as they allow the
highest spotting densities. In general, higher spotting
densities are advantageous in large scale screening and,

hence, preferred. As the person skilled in the art will
realise, the choice of said carrier will largely depend on
the host cell system under investigation.

The selective media used for growth of appropriate clones may
be in liquid or in solid form. Preferably, said selective
media when used in conjunction with a spotting robot and
membranes as planar carriers are solidified with agar on
which said spotted membranes are subsequently placed.
Alternatively, and also preferably, said selective media when
in liquid form are held within microtiter plates and said
transfer is made by replication.

Referring now to step (F) of the method of the invention, in
a preferred embodiment the activation state of the readout
system can be analysed by a variety of means. For example, it
can be analysed by visual inspection, radioactive,
chemiluminescent, fluorescent, photometric, spectrometric,
infrared, colourimetric or resonant detection.

More preferably, said identification in step (F) of host
cells that express interacting fusion proteins from
consideration of the activation state of said readout system
of host cells grown on the selective medium as specified in
step (F) is effected or assisted by using visual means.

Most preferably, said identification of host cells that
express interacting fusion proteins in step (F) from
consideration of the activation state of said readout system
is effected or assisted by automated digital image capture,
storage, analysis or processing. Here, automation stands for
the use of electronic devices such as computers in
conjunction with complex instruction sets such as software,
commercially available or self devised, which performs or
assists in performing large numbers of calculations on images
converted to a digital format. In this embodiment, positive
clones which are preferably arrayed on a planar carrier such

as a membrane are identified by comparison of digital images
obtained from the carrier after activation of said readout
system on said selective media specified in (E).

The analysis of a small number of clones or grids for
activity of the readout system can be conducted by manual
inspection of the activation state for each individual clone.
However, when dealing with the number of clones generated by
library vs. library interactions screens, or when analysing
regular grid patterns produced at densities presented here,
such manual inspection becomes time consuming to the extent
of almost being impossible.

According to the invention it is possible to efficiently
analyse regular grid patterns of 2H clones using visual
means. Thus, when members comprising an interaction are
identified, a digital image of the planar carrier is obtained
and analysis is effected by digital image capture, storage,
processing or analysis using an automated or semi-automated
image analysis system, such as described in Lehrach et al.
(1997). There are many forms and combinations of steps in
handling digital image data that the person skilled in the
art would know to apply to this task laid out in the present
invention.

Referring to step (G) of the present invention,
identification of the at least one member of the pair or
complex of interacting molecules may be effected by a variety
of means. In a preferred embodiment of the present invention,
at least one member of said pair or complex of interacting
molecules is characterised by nucleic acid hybridisation,
oligonucleotide hybridisation, nucleic acid or protein
sequencing, restriction digestion, spectrometry or antibody
reactions, determining the genetic information encoding said
at least one member. Once the first member of an interaction
has been characterised, the second member or further members
can also be characterised by any of the above methods.

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Preferably the identification of at least one member of an
interaction is effected by nucleic acid hybridisation,
antibody binding or nucleic acid sequencing.

More preferably, said identification of at least one member
of said pair or complex interacting molecules is effected
using regular grid patterns of said at least one interacting
molecules or of said genetic information encoding said at
least one member or of said genetic information encoding said
at least one member. Yet more preferably, construction of
said regular grid patterns in step (G) is effected or
assisted by automation. Yet more preferably, said automation
in step (G) is effected by an automated spotting, pipetting
or micropipetting device. Yet more preferably, said
automation in step (G) is implemented by employing a spotting
robot, spotting tool, pipetting system or micropipetting
system. Yet more preferably, said identification is effected
by automated digital image capture, storage, processing
and/or analysis. Yet more preferably, said nucleic acid
molecules, prior to said identification in step (G), are
amplified by PCR or are amplified in a different host cell as
a part of said genetic elements, more preferably in bacteria
and most preferably in E. coli.

If nucleic acid hybridisation is to be carried out, the
nucleic acid molecules comprised in the host cell and
encoding for at least one of the interacting molecules is
preferably affixed to a planar carrier. As is well known in
the art, said planar carrier to which said nucleic acid may
be affixed, can be for example a Nylon-, nitrocellulose- or
PVDF membrane, glass or silica substrate (DeRisi et al. 1996;
Lockhart et al. 1996). Said host cells containing said
nucleic acid may be transferred to said planar carrier and
subsequently lysed on the carrier and the nucleic acid
released through said lysis is affixed to the same position
by appropriate treatment. Alternatively, progeny of the host
cells may be lysed in a storage compartment and the crude or

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purified nucleic acid obtained is then transferred and
subsequently affixed to said planar carrier. Advantageously,
said nucleic acids are amplified by PCR prior to transfer to
the planar carrier. Most preferably said nucleic acid is
affixed in a regular grid pattern in parallel with additional
nucleic acids representing different genetic elements
encoding interacting molecules. As is well known in the art,
such regular grid patterns may be at densities of between 1
and 50 000 elements per square centimeter and can be made by
a variety of methods. Preferably, said regular patterns are
constructed using automation or a spotting robot such as
described in Lehrach et al. (1997) and Maier et al. (1997)
and furnished with defined spotting patterns, barcode reading
and data recording abilities. Thus it is possible to
correctly and unambiguously return to stored host cells
containing said nucleic acid from a given spotted position on
the planar carrier. Also preferably, said regular grid
patterns may be made by pipetting systems, or by
microarraying technologies as described by Shalon et al.
(1996), Schober et al (1993) or Lockart et al. (1996).
Identification is, again, advantageously effected by nucleic
acid hybridisation.

Once produced, nucleic acids carried on these arrays can be
detected using a variety of methods. Preferably, this method
is hybridisation using labelled hybridisation probes.
However, other detection methods such as mass-spectrometry
may be employed. Said labelled hybridisation probes can be
labelled with any detectable moiety including radioactive
elements, fluorescent and chemiluminescent molecules, or
molecules that can be detected via secondary enzymatic or
binding assays. Said hybridisation probe can comprise DNA,
RNA or PNA molecules, and may consist of a single class of
molecule,for example a short oligonucleotide, gene fragment,
cDNA clone, genomic fragment or YAC. Also, said hybridisation
probe may be a complex mixture of nucleic acids representing
the gene-expression state of a given tissue, cell type, or

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developmental or disease state. Two said complex mixtures of
nucleic acids may be used in two separate hybridisation
experiments with replica nucleic acid arrays to identify
those interactions that are specific or more commonly found
in the expression state of a given tissue compared to a
reference tissue. The methods of producing said complex
mixtures and their application as hybridisation probes to
nucleic acid arrays are well known in the art (for example,
Gress et al., 1996, Lockhart et al., 1996; De Risi et al.,
1996). This approach may be applicable to identify disease
specific protein-protein interactions that may be targeted by
therapeutic agents directed at said disease-specific protein-
protein interaction.

Using a detectable nucleic acid probe of interest, homologous
nucleic acids which are affixed on the planar carrier can be
identified by hybridisation. From the spotted position of
said homologous identified nucleic acid on the planar
carrier, the corresponding host cell in the storage
compartment can be identified which contains both or all
members of the interaction. The for example second member of
the interaction can now be identified by any of the above
methods. For example, by use of a radioactively labeled Ras
probe, homologous nucleic acids on the planar carrier can be
identified by hybridisation. The Ras interacting proteins can
now be identified from the corresponding host cell that
contains both the first genetic element homologous to the Ras
probe and the second genetic element encoding for these Ras
interacting proteins.

If multiple oligonucleotide hybridisations are carried out on
the nucleic acids affixed to the planar carrier, oligo
fingerprints of all genetic elements encoding the interacting
proteins can be obtained. These oligo fingerprints can be
used to identify all members of the interactions or those
members that belong to specific gene families, as described
in Maier et al. (1997).

If nucleic acid sequencing is used, the nucleic acid
molecules that encode the interacting proteins are, prior to
the identification in step (G), amplified by PCR or in said
genetic elements in host cells and preferably in *E. coli*.
Amplification of said genetic elements is conducted by
multiplication of the *E. coli* cells and isolation of said
genetic elements. Methods of identifying the nucleic acids
that encode interacting proteins by DNA sequencing and
analysis are well known in the art. By amplifying and
sequencing the nucleic acids that encode for both or all
members of an interaction from the same clone, the identity
of both or all members of the interaction can be determined.

If a specific antibody is to be used to determine whether a
protein of interest is expressed as a fusion protein within
an interaction library, it is advantageous to affix all
fusion proteins expressed from the interaction library onto a
planar carrier. For example, clones of the interaction
library that express fusion proteins can be transferred to a
planar carrier using a spotting robot as described in Lehrach
et al (1997). The clones are subsequently lysed on the
carrier and released proteins are affixed onto the same
position. Using, for example, an anti-HIP1-antibody (Wanker
et al. 1997), clones from the interaction library that
contain HIP1 fusion proteins and an unknown interacting
fusion protein can be identified. The unknown member of the
interacting pair of molecules can now be characterised from
the corresponding host cell by any of the above methods. The
antibodies used as probes may be directly detectably
labelled. Alternatively, said antibodies may be detected by a
secondary probe or antibody which may be specific for the
primary antibody. Various alternative embodiments using, for
example, tertiary antibodies may be devised by the person
skilled in the art on the basis of his common knowledge.

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It would be theoretically possible to systematically identify
all the members comprising the interactions using the methods
described above for all positive clones. However, this would
be very laborious, costly, and would cause many identical
interactions to be identified repetitively. It is likely that
any protein-protein interaction pathways would only be
developed stochastically as the relevant interactions were
randomly identified during the identification process.

Alternatively, the present invention provides for a method to
characterise the positive clones identified in a 2H search in
a more focused approach, preferably identifying directly
yeast clones that express interactions representing the next
step in an interaction network from the knowledge of a first
molecule that interacts with a given molecule, and hence
reduce the time, amount and cost of identifying the
interacting members by, for example, systematic DNA
sequencing.

Previously, a focused approach could only be followed within
the framework of the standard 2H techniques. For example,
starting with a gene of interest, a classic single bait 2H
experiment would be conducted to identify clones that
activated the readout system. These clones would subsequently
be tested to determine if they were positive or false
positive clones and the interacting members expressed in the
positive clones identified. The gene expressing a protein
identified as interacting with the initial bait of interest,
would then be sub-cloned and subjected to a second yeast-two
hybrid experiment to identify which further proteins it
interacted with. A separate 2H experiment would have to be
conducted for each separate protein-protein interaction step
in the pathway. Each step in such a sequential yeast 2H
approach would take over two weeks, and thus to generate
complete or even partially complete interaction pathways by
such an approach would be very time and cost consuming.

A modified hybridisation approach from the ones known in the art (Lennon, Lehrach, 1991; Ross et al., 1992; Shalon et al., 1996; Lehrach et al., 1997) is provided by the present invention. This approach is advantageous when applied to the identification of interacting members within the yeast two hybrid system. By hybridising a probe representing the gene of interest to a regular grid pattern of the nucleic acids including those that express the interacting members, the identification efforts can be focused only on those positive clones which hybridised to the probe of interest. This is because, as well as expressing the gene of interest, such hybridisation-positive and interaction-positive clones would also express a second, interacting protein encoded by one of the 2H vectors. By isolation of the plasmids carried from these hybridisation-positive clones from a stored copy of the interaction library and subjecting them to further characterisation procedures, the identification of proteins that interact with the gene of interest, sequential identification procedures can be focused on. For each step in the protein-protein interaction pathway to be investigated, this approach simply requires nucleic acid hybridisation, plasmid isolation, DNA sequencing and a second hybridisation using the isolated insert. Such a combination of standard procedures may be conducted within a matter of days, and several different pathways may be investigated in parallel by the use of replica nucleic acid arrays. Therefore, the time taken to investigate a given protein-protein interaction pathway is considerably shorter than by alternative approaches.

There are a number of further advantages of this hybridisation approach. Firstly, it provides an internal control as the clone from which the probe was isolated should be a hybridisation-positive clone. Secondly, the hybridisation approach may be used not only to identify those clones expressing interacting fusion proteins of interest, but also to ignore those clones that express fusion protein

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for which the investigator has no interest. For example, it
is known that some proteins (for example heat shock proteins)
are especially 'sticky', and generate positive clones in the
yeast 2H system that may have little biological relevance.
Positive clones expressing such `fortuitous' interactions may
be identified and hence ignored from further analysis by a
simple hybridisation to an array representing the DNA
encoding the fusion proteins expressed within cells of the
interaction library. Finally, if both members of a given
interaction have been identified, then it may be that the
investigator does not wish to waste further resources on re-
isolating the same interaction. Identifying those clones from
the interaction library that are hybridisation-positive for
both members of a previously identified interaction will
enable the investigator to ignore these clones for further
work. These embodiments have the advantage of saving the
investigator both cost and time. By careful selection of the
hybridisation probe and focusing only on hybridisation-
positive clones, the investigator can successively narrow his
search to those clones which carry coding regions, thereby
avoiding isolation of large numbers of short peptide
fragments caused by translation of 5' or 3' regions of genes.

The focused hybridisation approach to identifying
interactions based on hybridisation will rapidly identify
many interactions making up a protein-protein interaction
pathway. Indeed, by identifying most interactions that make
up several different protein-protein interaction pathways, it
will be extremely probable that two or more pathways will be
found to have a particular protein in common. Such pathways
can then be combined and hence form part of a network of
protein-protein interactions. Therefore, because this
approach can efficiently investigate several different
protein-protein pathways in parallel, it is highly suitable
to the generation of a network of protein-protein
interactions.

In a further preferred embodiment, the present invention
provides for a method further comprising:

(H)   providing at least one of said genetic elements in
    step (A), which additionally comprises or comprise a
    counterselectable marker, wherein said counterselectable
    markers are different for each type of genetic element;

(I)   selecting for interaction by transferring host
    cells or progeny of host cells, which transfer is
    optionally effected or assisted by automation in a
    regular grid pattern, in step (E) to

(viii)   at least one selective medium that allows
    growth of host cells only in the absence of a
    counterselectable marker specified in (H) and in the
    presence of a selectable marker; and

(ix)   a further selective medium that allows
    identification of host cells upon activation of the
    readout system;

    (J) identifying host cells in step (F) that contain
    interacting molecules that:

(x)   do not activate said readout system on said
    at least one selective medium specified in (i), and

(xi)   activate said readout system on said
    selective medium specified in (ii).

In a more preferred embodiment, said genetic element that
additionally comprises a counterselectable marker further
specifies an activation domain fusion protein.
As referred to above, false positive clones have proven to
dramatically reduce the overall usefulness of the 2H system.
For example, by inclusion of a marker counterselecting for
the absence of a genetic element that specifies one of a pair
of the potentially interacting partners, clones that will
grow and therefore only carry the second genetic element
specifying the second partner can now be tested for the
activation of the readout system. If the clone containing
only the fusion protein encoded by the second genetic element

activates the readout system in the absence of the other genetic element, then it will be classified as a false positive. Thus, only clones that activate the readout system in the presence of both or all genetic elements, but do not activate the read out system when one of the genetic elements is lost are classified as positives. In order to save time and effort, preferably only the plasmid encoding the activation domain is removed, as the fusion protein comprising the DNA binding domain is more likely to have auto-activating properties.

In a further preferred embodiment, the present invention provides for a method further comprising:

(K) providing at least two of said genetic elements in step (A), which additionally comprise different counterselectable markers;

(L) selecting for interaction by transferring host cells or progeny of host cells in step (E) to

  (v) at least one selective medium, wherein said selective medium precludes growth of host cells in the presence of the first counterselectable marker of the counterselectable markers specified in (K) and allows growth in the presence of a first selectable marker;

  (vi) at least one selective medium, wherein said selective medium precludes growth of host cells in the presence of the second counterselectable marker of the counterselectable markers specified in (K) and allows growth in the presence of a second selectable marker;

  (vii) a further selective medium that allows identification of said host cells upon activation of the readout system; and

(M) identifying host cells that contain molecules that:

(viii)   do not activate said readout system on said at
          least one selective medium specified in (v); and

(ix) do not activate said readout system on said at least one
      selective medium specified in (vi); and

(x)   activate said readout system on said selective medium
      specified in (vii).

In a more preferred embodiment, said at least two genetic
elements that additionally comprise a counterselectable
marker further specify a DNA binding domain fusion protein
and an activation domain fusion protein, respectively.

Yet more preferably, said counterselectable marker or
counterselectable markers of step (H) or (K) are selected
from the group of URA3, LYS2, sacB, CAN1, CYH2, rpsL, or
lacY.

Additionally preferred is an embodiment, wherein the transfer
of host cells or progeny of host cells in step (I) or (L) is
effected or assisted by automation. More preferably, said
automation in step (I) or (L) is effected by an automated
replicating, picking, spotting, pipetting or micropipetting
or cell sorting device. Most preferably, said automation in
step (I) or (L) is implemented by employing a replicating
robot, picking robot, spotting robot, spotting tool,
automated pipetting, micropipetting system, or fluorescent
assisted cell sorting (FACS) system.

Herein, the same test is also applied to the first genetic
element, counterselecting for the absence of the second
genetic element. When employing the present invention
according to this embodiment, only clones that activate the
readout system in the presence of both or all genetic
elements, but do not activate the read out system when either
of the genetic elements is lost are classified as positives.

By removing both genetic elements, a maximum number of false
positives can be identified. This becomes particularly useful
with growing total numbers of clones.

The use of the counterselectable system described in this
invention compared to the prior art has the advantage that
only one strain which expresses the potentially interacting
fusion proteins is generated and must be analysed. In
contrast, to detect false positive clones using the state of
the art yeast 2H system, plasmids that encode fish proteins
usually need to be isolated and retransformed into yeast
cells harboring plasmids that encode unrelated bait proteins.
Further, the enormous number of false positive clones that
would be isolated when using the classical 2H system on a
large scale, yet are discriminated by the method of this
invention no longer precludes an effective high through-put
analysis of clones. In the long run, it is expected that the
method of the present invention is especially advantageous
for a high throughput analysis of a large number of clones
containing interacting molecules since many specific
interactions and the individual members of these interactions
can be identified in a parallel and automated approach.

A significant advantage of the method of invention over
existing yeast two-hybrid systems is the scale at which such
identification of interactions and interaction members can be
made. Preferably, the method of invention screens library vs.
library interactions using arrayed interaction libraries.
Hence, in one preferred embodiment of the present invention,
said genetic information specifying one of said potentially
interacting molecules is different for each host cell in a
set of host cells or a majority of host cells in a set of
host cells.

In a particularly preferred embodiment, said genetic
information specifying one of said potentially interacting
molecules is identical in not more than 10 %, preferably not

more than 5 %, more preferably not more than 2 %, most
preferably not more than 1 % of host cells in a set of host
cells.

The present invention also relates to a method for the
production of a pharmaceutical composition comprising
formulation of said at least one member of said pair or
complex of interacting molecules identified by the method of
the invention in a pharmaceutically acceptable form. Said
pharmaceutical composition comprises at least one of the
aforementioned compounds identified by the method of the
invention, either alone or in combination, and optionally a
pharmaceutically acceptable carrier or excipient. Examples of
suitable pharmaceutical carriers are well known in the art
and include phosphate buffered saline solutions, water,
emulsions, such as oil/water emulsions, various types of
wetting agents, sterile solutions etc. Compositions
comprising such carriers can be formulated by conventional
methods. These pharmaceutical compositions can be
administered to subject in need thereof at a suitable dose.
Administration of the suitable compositions may be effected
by different ways, e.g., by intravenous, intraperitoneal,
subcutaneous, intramuscular, topical or intradermal
administration. The dosage regimen will be determined by the
attending physician and other clinical factors. As is well
known in the medical arts, dosages for any one patient
depends upon many factors, including the patient's size, body
surface area, age, the particular compound to be
administered, sex, time and route of administration, general
health, and other drugs being administered concurrently.
Dosages will vary but a preferred dosage for intravenous
administration of DNA is from approximately 10^6 to 10^{22}
copies of the nucleic acid molecule. Proteins or peptides may
be administered in the range of 0,1 ng to 10 mg per kg of
body weight. The compositions of the invention may be
administered locally or systematically. Administration will
generally be parenterally, e.g., intravenously; DNA may also

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be administered directly to the target site, e.g., by
biolistic delivery to an internal or external target site or
by catheter to a site in an artery.

The present invention further relates to a method for the
production of a pharmaceutical composition comprising
formulating an inhibitor of the interaction of the at least
one member of said pair or complex of interacting molecules
identified by the method of the invention with another
molecule, preferably also identified by the method of the
invention, in a pharmaceutically acceptable form. The
inhibitor may be identified according to conventional
protocols. Additionally, molecules that inhibit existing
protein-protein interactions can be isolated with the yeast
2H system using the URA3 readout system. Yeast cells that
express interacting GAL4ad and LexA fusion proteins which
activate the URA3 readout system are unable to grow on
selective medium containing 5-FOA. However, when an
additional molecule is present in these cells which disrupts
the interaction of the fusion proteins the URA3 readout
system is not activated and the yeast cells can grow on
selective medium containing 5-FOA. Using this method
potential inhibitors of a protein-protein interaction can be
isolated from a library comprising these inhibitors. Systems
corresponding to the URA3 system may be devised by the person
skilled in the art on the basis of the teachings of the
present invention and are also comprised thereby.

Also, the present invention relates to a method for the
production of a pharmaceutical composition comprising
identifying a further molecule in a cascade of interacting
molecules, of which the at least one member of interacting
molecules identified by any of the above methods is a part of
or identifying an inhibitor of said further molecule. Once at
least one member of the interacting molecules has been
identified, it is reasonable to expect that said member is a
part of a biological cascade. Identification of additional

members of said cascade can be effected either by applying
the method of the present invention or by applying
conventional methods. Also, inhibitors of said further
members can be identified and can be formulated into
pharmaceutical compositions.

Moreover, the invention relates to a kit comprising:

(N)   Host cells, comprising a readout system which allows
      host cells to be counterselected against auto-activation
      of said readout system; and

(O)   at least one genetic element comprising a selectable
      marker, a counterselectable marker and genetic
      information encoding an activation domain or a DNA
      binding domain, which activation domain and DNA binding
      domain are together able to activate said readout
      system;

wherein said host cells are not yeast cells.

In a preferred embodiment, said kit comprises bacterial
cells.

Such kits could be used to carry out the first aspect of the
invention.

Futhermore, the invention relates to a Kit comprising:

(P)   Host cells comprising a readout system which allows host
      cells to be visually differentiated upon activation of
      said readout system; and

(Q)   at least one genetic element comprising a selectable
      marker and genetic information encoding an activation
      domain or a DNA binding domain, which activation domain

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and DNA binding domain are together able to activate
said readout system;

In a preferred embodiment, said kit comprises bacterial cells
or mammalian cells.

Such kits could be used to carry out the second aspect of the
invention.

These kits could be used, for example, for the rapid
identification of inhibitors of interactions or pathways of
interactions, for the identification of pathways that toxic
substances act on, or, concomitantly, detoxifying agents and
for the identification of interaction pathways.

(R)   The invention further relates to a method for the
         identification of at least one member of a pair or
         complex of interacting molecules, comprising:

(S)   providing at least two sets of host cells each
         containing at least one genetic element with a
         selectable marker different for each set of host cells,
         said genetic elements each comprising genetic
         information specifying one of said molecules, said host
         cells further carrying a readout system that is
         activated upon the presence of autoactivating molecules;

(T)   screening or selecting against molecules that auto-
         activate said readout system by transferring progeny of
         at least one set of host cells to:

(U)   selecting for said interaction by transferring
         progeny in a regular grid pattern effected by automation
         to:

(xi)   a selective medium which allows growth of
                  said host cells in the presence of said
                  selectable marker different for each set of
                  host cells and which precludes growth of

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said host cells upon auto-activation of
said readout system; or; and/or

(xii)   a selective medium which allows growth of
                all of said host cells and visual
                differentiation between those cells whose
                readout system has been auto-activated and
                those whose readout system has not been
                auto-activated;

(K)   combining in host cells said genetic elements from
           at least two different sets of host cells, wherein
           at least one set of host cells grows on said
           selective medium specified in (xi) or does not
           auto-activate said readout system on said selective
           medium specified in (xii);

(L)   allowing at least one interaction, if any, to occur;

(M)   identifying host cells obtained in step (c)
           containing interacting molecules that activate said
           readout system:

(N)   identifying at least one member of said pair of
           interacting molecules.

Preferably, the data obtained by using the method of the
present invention can be accessed through the use of software
tools or graphical interfaces that enable to easily query the
established interaction network with a biological question or
to develop the established network by the addition of further
data. A computer-based system provides a robust and efficient
solution for handling the large amount of protein-protein
interaction data produced by the method of the invention.

Such a solution would include the features of simple data-
entry procedures, efficient use of memory and disk space for
storage and data-processing, the ability to communicate and
utilise with different data-bases and/or computers across
intra or internets, interfaces to allow querying of the data-
base by an investigator and visual display of the results of

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the query. Relational or object orientated data-bases, with
data-parsing and display programs supporting said data-base
are possible embodiments to implement said solution. As way
of example, Figure 2 displays a scheme and features for a set
of data-tables suitable for managing such interaction data
that could be implemented in either relational or object-
orientated data-bases. The primary links between table-keys
are indicated, as are the suggested fields or elements to be
held within each table. It would be possible that certain
elements of a table may be expanded into an additional table
holding further data. Likewise, it would be possible that
certain tables may be expanded into an additional data-base
to hold and manage further data. Said additional data-base
may be held on the same, or remote computers. Elements of the
table may be recorded in numerical, descriptive of fixed
format as may be appropriate for the data, and to provide
efficient querying, it is of advantage that where
appropriate, elements are recorded in controlled vocabulary.
Figure 3 displays in what part of the work process during an
interaction experiment each table is most relevant and where
it forms the underlying data-set from which work-flow
management software for that part of the process is based.

Other computer-based methods of generating visual
representations of specific interactions, partial or complete
protein-protein interaction networks can be employed to
automatically calculate and display the required interactions
most efficiently. As is well known in the art, computer data-
bases are a valuable resource for large-scale biological and
molecular biological research.

An established computer data-base of protein interactions has
many useful applications. For example, it may be used to
predict the existence of new biological interactions or
pathways, or to determine links between biological networks.
Furthermore with this method, the function and localisation
of previously unknown proteins can be predicted by

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determining their interaction partners. It also can be used
to predict the response of a cell to changes in the
expression of particular members of the networks without
making a molecular, cellular or animal experiment. Finally,
these data can be used to identify proteins or interactions
between proteins within a medically relevant pathway, which
are suitable for therapeutic intervention, diagnosis or the
treatment of a disease (Figure 1).

In summary, a significant advantage of the method of
invention over existing 2H systems is the ease of removal of
false positives from sets of host cells designed for 2H
screening experiments. A further advantage lies in the scale
at which such identification of interactions and interaction
members can be made. Due to the ease of automating the method
of the invention at different stages, fast and reliable
screening of large numbers of clones will be possible.

The figures show:

Figure 1
The applications of an established and exhaustive network of
protein-protein interactions. The identity of positive clones
and the identity of the members comprising the interactions
for the entire interaction library are stored in a database.
These data are used to establish a network of protein-protein
interactions which can be used for a variety of purposes. For
example, to predict the existence of new biological
interactions or pathways, or to determine links between
biological networks. Furthermore with this method, the
function and localisation of previously unknown proteins can
be predicted by determining their interaction partners. It
also can be used to predict the response of a cell to changes
in the expression of particular members of the networks.
Finally, these data can be used to identify proteins within a
medically relevant pathway which are suitable for

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therapeutic, diagnosis intervention and for the treatment of
disease.

Figure 2
A scheme and features for a set of data-tables suitable for
storing, managing and retrieving data from a large-scale
protein-protein interaction screen. The scheme could be
implemented in either relational or object-orientated data-
bases. The primary links between table-keys are indicated, as
are the suggested fields or elements to be held within each
table.

Figure 3
A process flow representing the experimental and informatic
flow during a large-scale protein-protein interaction screen.
The figure displays in which part of the experimental steps
each table from a the data-base described above is most
applicable. Each table forms the underlying data-set from
which work-flow management software for that part of the
process is based.

Figure 4
Plasmids constructed for the improved 2-hybrid system.

The plasmid maps of the pBTM118a, b and c DNA binding domain
vector series and the pGAD428a, b and c activation domain
vector series. Both plasmids contain the unique restriction
enzyme sites for *Sal* I and *Not* I which can be used to clone a
genetic fragment into the multiple cloning site. The plasmids
are maintained in yeast cells by the selectable markers TRP1
and LEU2 respectively. The loss of the plasmids can be
selected for by the counterselective markers CAN1 and CYH2
respectively.

Polylinkers used within the multiple cloning site to provide
expression of the genetic fragment in one of the three
reading frames.

Figure 5

The structure of the URA3 readout system carried by the
plasmid pLUA. Important features of pLUA include the URA3
gene which is under the transcriptional control of the
lexAop-GAL1 promoter, the *ADE2* selectable marker that allows
yeast *ade2*-auxotrophs to grow on selective media lacking
adenine and the ß-lactamase gene (bla) which confers
ampicilin resistance in *E.coli*. The pLUA plasmid replicates
autonomously both in yeast using the 2μ replication origin
and in *E.coli* using the ColE1 origin of replication.

Figure 6

A schematic overview of one embodiment of the method of the
invention. For the parallel analysis of a network of protein-
protein interactions using the method of the invention, a
library of plasmid constructs that express DNA binding domain
and activation domain fusion proteins is provided. These
libraries may consist of specific DNA fragments or a
multitude of unknown DNA fragments ligated into the improved
binding domain and activating domain plasmids of the
invention which contain different selectable and
counterselectable markers. Both libraries are combined within
yeast cells by transformation or interaction mating, and
yeast strains that express potentially interacting proteins
are selected on selective medium lacking histidine. The
selective markers TRP1 and LEU2 maintain the plasmids in the
yeast strains grown on selective media, whereas CAN1 and CYH2
specify the counter-selectable markers that select for the
loss of each plasmid. HIS3 and lacZ represent selectable
markers in the yeast genome, which are expressed upon
activation by interacting fusion proteins. The readout system
is, in the present case, both growth on medium lacking
histidine and the enzymatic activity of ß-galactosidase which
can be subsequently screened. A colony picking robot is used
to pick the resulting yeast colonies into individual wells of
384-well microtiter plates, and the resulting plates are

incubated at 30°C to allow cell growth. The interaction
library held in the microtiter plates optionally may be
replicated and stored. The interaction library is
investigated to detect positive clones that express
interacting fusion proteins and discriminate them from false
positive clones using the method of the invention. Using a
spotting robot, cells are transferred to replica membranes
which are subsequently placed onto one of each of the
selective media SD-leu-trp-his, SD-leu+CAN and SD-trp+CHX.
After incubation on the selective plates, the clones which
have grown on the membranes are subjected to a ß-Gal assay
and a digital image from each membrane is captured with a CCD
camera which is then stored on computer. Using digital image
processing and analysis clones that express interacting
fusion proteins can be identified by considering the pattern
of ß-Gal activity of these clones grown on the various
selective media. The individual members comprising the
interactions can then be identified by one or more
techniques, including PCR, sequencing, hybridisation,
oligofingerprinting or antibody reactions.

Figure 7
A schematic overview of one embodiment of the method of the
invention. For the parallel analysis of a network of protein-
protein interactions with the method of the invention, two
libraries of plasmid constructs that express DNA binding
domain or activation domain fusion proteins are provided.
These libraries may consist of specific DNA fragments or a
multitude of unknown DNA fragments ligated into binding
domain and activating domain plasmids which contain the
selectable markers TRP1 and LEU2, an doptionally the
counterselective markers CAN1 and CYH2 respectively. The
libraries are transformed into either _Mata_ or _Mata_ yeast
strains containing the URA3 readout system and are
subsequently plated onto selective media containing 5-
fluoroorotic acid (5-FOA). Only those yeast cells that
express fusion proteins unable to auto-activate the URA3

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readout system will grow in the presence of 5-FOA. The
resulting yeast strains that express only non-auto-activating
proteins can then be directly used in an automated
interaction mating approach to generate ordered arrays of
diploid strains which can be assayed for activation of the
lacZ readout system. a) Individual yeast cells that express
single fusion proteins unable to activate the URA3 readout
system are transferred into wells of a 384-well microtiter
plate using a modified picking robot. The yeast strains held
in the microtiter plates can optionally be replicated and
stored. The microtiter plates contain a growth medium lacking
amino acids appropriate to maintain the corresponding
plasmids in the yeast strains. The interaction matings are
subsequently performed by automatically transferring a *Mata*
and a *Matα* yeast strain to the same position on a Nylon
membrane using automated systems as described by Lehrach *et
al.* (1997). Alternatively, a pipetting or micropipetting
system (Schober *et al.* 1993) can be used to transfer small
volumes of individual liquid cultures of a yeast strain onto
which a lawn of yeast cells derived from at least one yeast
clone of the opposite mating type is sprayed or applied.
Yeast strains may be applied singly or as pools of many
clones. By both methods ordered arrays of yeast clones are
incubated overnight at 30°C to allow interaction mating to
occur. The resulting diploid cells are then analysed in a ß-
Gal assay as described by Breeden & Nasmyth (1985). b) Yeast
strains that grew on selective media containing 5-FOA are
pooled and interaction mating between the *Mata* and *Matα*
strains is made within liquid YPD medium. Those diploid yeast
strains that express interacting proteins are selected by
plating on selective medium lacking histidine and uracil. The
selective markers TRP1 and LEU2 maintain the plasmids in
yeast strains grown on selective media. HIS3, URA3 and lacZ
represent reporter genes in the yeast cells, which are
expressed on activation by interacting fusion proteins. The
readout system is, in the present case, growth on medium
lacking histidine and/or uracil and enzymatic activity of ß-

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galactosidase which can be screened at a later time point. A
modified colony picking robot is used to pick the diploid
yeast colonies into individual wells of 384-well microtiter
plates containing selective medium, and the resulting plates
are incubated at 30°C to allow cell growth. The interaction
library optionally may be replicated and stored. Using a
spotting robot, diploid cells are transferred to replica
membranes which are subsequently placed onto growth medium.
Optionally, replica mambranes can be placed on the
counterselective media SD-trp+CHX or SD-leu+CAN. The
resulting regular arrays of diploid yeast clones are analysed
for ß-Gal activity as described by Breeden & Nasmyth (1985).
In either case a) and b), a digital image from each dried
membrane is captured with a CCD camera which is then stored
on computer. Using digital image processing and analysis
clones that express interacting fusion proteins can be
identified by considering the ß-Gal activity of these clones
spotted in a defined pattern grown the membranes placed on
the variious selective media. The individual members
comprising the interactions can then be identified by one or
more techniques, including PCR, sequencing, hybridisation,
oligofingerprinting or antibody reactions.

Figure 8
Predicted interactions between fusion proteins used to create
the defined interaction library. The fusion proteins enclosed
with dark rounded boxes are believed to interact as shown.
The LexA-HIP1 and GAL4ad-LexA fusion proteins enclosed by
thin rectangular boxes have been shown to activate the LacZ
readout system without the need for any interacting fusion
protein. The two proteins LexA and GAL4ad, and the three
fusion proteins GAL4ad-HIPCT, GAL4ad-14-3-3 and LexA-MJD (all
unboxed) are believed not to interact with each other or
other fusion proteins used in this example.

Figure 9

Identification of positive clones that contained interacting
fusion proteins from false positive clones using the method
of the invention. Three different yeast clones each
containing pairs of plasmid constructs (positive control:
pBTM117c-SIM1 & pGAD427-ARNT; negative control: pBTM117c &
pGAD427 and false-positive control: pBTM117c-HIP1 & pGAD427)
were transferred by hand to four agar plates each containing
a different selective medium (SD-leu-trp, SD-leu-trp-his, SD-
leu+CAN and SD-trp+CAN), and incubated for 48 hours at 30 °C.
The yeast colonies were subsequently transferred to a Nylon
membrane and assayed for β-gal activity by the method of
Breeden and Nasmyth (1985).

Figure 10

Digital images of the ß-gal assays made from the replica
Nylon membranes containing the defined interaction library
obtained from the selective media (a) SD-leu-trp-his, (b) SD-
trp+CHX and (c) SD-leu+CAN. In each case, The left hand side
of each membrane contains control clones and clones from the
defined interaction library, and the right hand side contains
only clones from the defined interaction library. The two
regions marked on the first membrane represent those clones
magnified in Figure 11. The overall size of each membrane is
22 x 8 cm and contains 6912 spot locations at a spotting
pitch of 1.4 mm.

Figure 11

Magnification of clones from the interaction library taken
from the same region of three membranes obtained from the
selective media SD-leu-trp-his, SD-trp+CHX and SD-leu+CAN
assayed for β-gal activity:

Clones imaged from a region of the right hand side of the
membrane containing the defined interaction library. Clones
from the defined interaction library that express interacting

proteins are ringed and correspond to the microtiter plate
addresses 06L22 and 08N24.

Clones imaged from a region of the left hand side of the same
membranes containing control clones and clones from the
interaction library, where clones around each ink guide-spot
are arranged as shown and correspond to: 00 Ink guide spot;
01 False positive control clone that expresses the fusion
protein GAL4ad-LexA; 02 False positive clone expressing the
fusion protein LexA-HIP1; 03 Positive control clone
expressing the interacting fusion proteins LexA-SIM1 &
GAL4ad-ARNT; 04 Clone from the defined interaction library.
The positive control clone (spot position 03) is ringed.

Figure 12
A subset of the list of clones identified by computer query
of data produced by automated image analysis and
quantification of the ß-galactosidase activity. Each record
represents the ß-galactosidase activity for a given clone
grown on three selective media. This program queried the data
to identify all clones from the interaction library that had
activated the reporter gene (score > 0) when grown on minimal
medium lacking, leucine, trptophan, and histidine (SD-leu-
trp-his), yet had not on either of the counterselective media
(score on both media equal to 0).

Two positive clones 06L22 and 08N24 characterised by
hybridisation are present within the computer file.

Figure 13
Characterisation by hybridisation of the genetic fragments
carried by the clones 06L22 and 08N24. A 1.3 kb, SIM1 and a
1.4 kb ARNT DNA fragment were used as nucleic acid probes for
hybridisation to high-density spotted membranes containing
DNA from the defined interaction library. These clones were
characterised as containing SIM1 and ARNT genetic fragments
by hybridisation. The images are of the same region of the

membranes as those shown in Figure 11 a. The spot positions
of the clones 06L22 and 08N24 are ringed.

Figure 14

Identification of the SIM1 and ARNT DNA fragments from the
yeast two hybrid plasmid carried by the clone 06L22 by duplex
PCR. Plasmid DNA was isolated from a liquid culture of the
clone 06L22 by a QiaPrep (Hilden) procedure and the inserts
contained within the plasmids were amplified by PCR using the
primer pairs, 5'-TCG TAG ATC TTC GTC AGC AG-3' & 5'-GGA ATT
AGC TTG GCT GCA GC-3' for the plasmid pBTM117c and 5'-CGA TGA
TGA AGA TAC CCC AC-3' & 5'-GCA CAG TTG AAG TGA ACT TGC-3' for
pGAD427. Lane 1 contains a Lamda DNA digestion with *Bst*EII as
size marker; Lane 2 contains the duplex PCR reaction from
plasmids isolated from clone 06L22; Lanes 3 and contain
control PCR amplifications from the plasmids pBTM117c-SIM1
and pGAD427-ARNT respectively.

Figure 15

Readout system ativation for clones in a regular grid pattern
from an interaction library. 23 384-well microtiter plates of
the sea urchin interaction library were spotted in a `3x3
duplicate' regular grid pattern around an ink guide-spot on a
222 x 222 mm porous membrane (Hybond N+, Amersham, UK) using
a spotting robot. The membrane was incubated in SD-leu-trp-
his medium for 3 days, assayed for lacZ expression using the
ß-gal assay as described by Breeden & Nasmyth (1985) and air
dried overnight. A digital image was captured using a
standard A3 computer scanner.

Figure 16

Hybridisation of a gene fragment (Probe A) encoding for
Protein A to an array of DNA from an interaction library. The
probe was labelled radioactively by standard protocols, and
hybridisation-positive clones from the interaction library
are identified by the automated image analysis system. The
position of clone 5K20, from which the gene fragment was

isolated, is indicted. Other hybridisation-positive clones
also carry this gene-fragment, and by recovery of interacting
members from these clones, a protein-protein interaction
pathway for Protein A can be uncovered.

Figure 17
A graphical representation of the hybridisation-positive
clones generated by hybridisation of Probe A to a DNA array
representing the interaction library.

Figure 18
A graphical representation of hybridisation- and interaction-
positive clones generated by a subsequent hybridisation with
probe B (isolated from the clone marked in a grey box). Also
shown, are the positions of the hybridisation-positive clones
from probe A. Interaction-positive clones that carry both
gene fragments are identified as hybridising with both
probes.

Figure 19
A graphical representation of hybridisation- and interaction-
positive clones generated by a further hybridisation with
probe C isolated from the clone 6D18 (marked by a grey box
and "B/C"). Also shown are the hybridisation signals for
probes A and B. By considering common hybridisation signals
for interaction-positive clones and subsequent DNA sequencing
of the inserts carried by these clones, protein-protein
interactions can be uncovered. The figure also shows an
interaction pathway uncovered between Proteins A, B an C
based on these data.

Figure 20
Automated visual differentiation of yeast cells expressing
single fusion proteins able to activate the LacZ readout
system. A defined library of L40ccu yeast clones expressing
different fusion proteins cloned in the plasmid pBTM117c was
plated onto minimal medium lacking tryptophan, buffered to pH

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7.0 with potassium phosphate and containing 2 ug/ml of X-Gal
(SD-trp/XGAL). White colonies that have not autoctivated the
LacZ reporter gene are automatically recognised and marked
with a red horizontal cross. A colony that has turned blue
due to expression of a single fusion protein able to auto-
activate the LacZ reporter gene is automatically recognised
due to its darker colour and the presence of a `hole'. An
arrow indicates this colony. All colonies unsuitable for
further analysis and picking (including those too small or
touching colonies) are automatically recognised and marked
with a blue diagonal cross.

Figure 21
Results of automated interaction mating to identify diploid
yeast strains that express interacting fusion proteins. a)
Progeny of the yeast strains x1a and x2a were spotted at
positions 1 and 2 on a nylon membrane using a spotting robot
such as described by Lehrach et al. (1997). The yeast strains
y1α and y2α of the opposite mating type were subsequently
spotted on positions 1 and 2 which already contained cells
from the strains x1a and x2a. To assist in recognition of the
duplicate spotting pattern, ink was spotted in position 2
directly to the right of the spotted yeast clones. b) The
membrane was transferred to a YPD agar plate and was
incubated at 30° C overnight to allow interaction mating to
occur. c) Diploid yeast cells that had grown on the membrane
were subsequently analysed for ß-galactosidase activity using
the method of Breeden & Nasmyth (1985).

Figure 22
The two vectors constructed to provide further genetic
features to enable the method of invention within a
prokaryotic two-hybrid system. The vectors are based on the
pBAD series of vectors which provide tight inductive-control
of expression of cloned genes using the promoter from the
arabinose operon (Guzman et al., 1995 J. Bact. 177: 4141-

4130), and can be maintained in the same *E.coli* cell by
virtue of compatible origins of replication.

The plasmid pBAD18-αRNAP expresses under the control of the
arabiose promoter, fusion proteins between the α amino
terminal domain (NTD) of the α-subunit of RNA polymerase and
DNA fragments cloned into the multiple cloning site. The
presence of this plasmid in kanamycin sensitive cells can be
selected by plating on growth medium supplemented with
kanamycin, or for its absence by the counterselective *rpsL*
allele by plating on media supplemented with streptomycin
(Murphy et al. 1995).

The plasmid pBAD30-cI expresses under the control of the
arabinose promoter, fusion proteins between the λcI protein
and DNA fragments cloned into the multiple cloning site. The
presence of this plasmid in amplicillin sensitive cells can
be selected by plating on growth medium supplemented with
amplicillin, or for its absence by the counterselective *lacY*
gene by plating on media supplemented with 2-nitrophenyl-ß-D-
thiogalactosidase (tONPG) (Murphy et al. 1995). Additionally,
the oriT sequence enables unidirectional genetic exchange of
the pBAD30-cI plasmid and its derivatives from E.coli cells
containing the F' fertility factor to F⁻ strains lacking the
fertility factor.

Examples

Example 1:   Construction of vectors yeast strains and
readout system for an improved yeast two-hybrid system

1.1 Construction of vectors

The plasmids constructed for an improved yeast two-hybrid
system pBTM118 a, b and c and pGAD428 a, b and c are shown in
Figure 4. Both sets of vectors can be used for the
construction of hybrid (fusion) proteins. The vectors contain

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the unique restriction sites *Sal* I and *Not* I located in the
multiple cloning site (MCS) region at the 3´- end of the open
reading frame for either the *lexA* coding sequence or the
GAL4ad sequence Figure 4b).

With both sets of plasmids fusion proteins are expressed at
high levels in yeast host cells from the constitutive *ADH1*
promoter (P) and the transcription is terminated at the *ADH1*
transcription termination signal (T). The two-hybrid plasmids
shown in Figure 4a are shuttle vectors that replicate
autonomously in both *E. coli* and *S. cerevisiae*.

The three plasmids pBTM118 a, b and c are used to generate
fusions of the LexA protein (amino acids 1-220) and a protein
of interest cloned into the MCS in the correct orientation
and reading frame. The plasmids pBTM118 a, b and c are
derived from pBTM117c (Wanker et al., 1997) by insertion of
the adapters shown in Table 1 into the restriction sites *Sal*
I and *Not* I to generate the improved vectors with three
different reading frames.

The plasmids pBTM118 a, b and c carry the wild type yeast
CAN1 gene for counterselection, which confers sensitivity to
canavanine in transformed yeast cells (Hoffmann, 1985). The
plasmids also contain the selectable marker *TRP1*, that allows
yeast trp1-auxotrophs to grow on selective synthetic medium
without tryptophan, and the selectable marker *bla* which
confers ampicillin resistance in *E. coli*.

The plasmids pGAD428 a, b and c are used to generate fusion
proteins that contain the GAL4 activation domain (amino acids
768-881) operatively linked to a protein of interest. The
plasmids pGAD428 a, b and c carry the wild type yeast *CYH2*
gene, which confers sensitivity to cycloheximide in
transformed cells (Kaeufer et al., 1983), the selectable
marker *LEU2*, that allows yeast leu2-auxotrophs to grow on
selective synthetic medium without leucine, and the bacterial

marker *aphA* (Pansegrau et al., 1987) which confers kanamycin
resistance in *E. coli*. The plasmids pGAD428a, b and c were
created from pGAD427 by ligation of the adapters shown in
Table 1 into the MCS to construct the improved vectors with
three different reading frames.

For the construction of pGAD427 a 1.2 kb *Dde* I fragment
containing the *aphA* gene was isolated from pFG101u (Pansegrau
et al., 1987) and was subcloned into the *Pvu* I site of the
pGAD426 using the oligonucleotide adapters 5'- GTCGCGATC-3'
and 5'-TAAGATCGCGACAT-3'. The plasmid pGAD426 was generated
by insertion of a 1.2 kb *Eco* RV *CYH2* gene fragment, which was
isolated from the pAS2-1 (Clonetech) into the *Pvu* II site of
pGAD425 (Han and Collicelli, 1995).

1.2 Construction of yeast strains

To allow for the improved yeast two-hybrid system, three
Saccharomyces cerevisiae strains L40cc, L40ccu and L40ccuα
were created. The *S. cerevisiae*. L40cc was created by site
specific knock-out of the CYH2 and CAN1 genes of L40
(Hollenberg et al., Mol. Cell. Biol. 15: 3813-3822), and
L40ccu created by site specific knock-out of the URA3 gene of
L40cc (Current Protocols in Molecular Biology, Eds. Ausubel
et al. John Wiley & Sons: 1992) The strain L40ccuα was
created by conducting a mating-type switch of the strain
L40ccu by standard procedures (Ray BL, White CI, Haber JE
(1991)). The genotype of the L40cc strain is: *Mata his3Δ200*
trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$*-HIS3*
URA3::(lexAop)$_8$*-lacZ GAL4 can1 cyh2*, The genotype of the
L40ccu strain is: *Mata his3Δ200 trp1-901 leu2-3,112 ade2*
LYS2::(lexAop)$_4$*-HIS3 ura3::(lexAop)*$_8$*-lacZ GAL4 can1 cyh2*, and
that of L40ccuα is *Matα his3Δ200 trp1-901 leu2-3,112 ade2*
LYS2::(lexAop)$_4$*-HIS3 ura3::(lexAop)*$_8$*-lacZ GAL4 can1 cyh2*.

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1.3 Readout system

Figure 5 shows the *URA3* readout system carried by the plasmid
pLUA. This *URA3* readout system under the control of a
bacterial LexAop upstream activation sequence (UAS) can be
used within the yeast 2-hybrid system both as a counter
selective reporter gene and as a positive selection reporter
gene to eliminate false positive clones. The plasmid contains
the features of the UAS_{lexAop}-URA3 readout system, the
selectable marker *ADE2* that allows yeast ade2-auxotrophs to
grow on selective media without adenine and the *bla* gene
which confers amplicillin resistance in *E.coli*. The plasmid
pLUA is a shuttle vector that replicates autonomously in *E.
coli* and yeast.

For the construction of pLUA a 1.5 kb *Sac* I/*Cla* I UAS_{lexAop}-
URA3 fragment was isolated from pBS-lexURA and ligated
together with a 2.4 kb *Sac* I/*Cla* I *ADE2* fragment into *Cla* I
digested pGAD425Δ. pBS-lexURA was generated by ligating *URA3*
fragment together with a UAS_{lexAop} fragment into pBluescript
SK+. The *URA3* and UAS_{lexAop} fragments were obtained by PCR
using genomic DNA from *S. cerevisiae* strain L40c using
standard procedures and anchor primers which gave rise to
complementary overhangs between the two consecutive fragments
which were subsequently anealed to generate the chimeric
sequence (see, for example, Current Protocolls in Molecular
Biology, Eds. Ausubel et al. John Wiley & Sons: 1992). The
ADE2 gene was isolated by PCR using genomic DNA from
SEY6210α. pGAD425Δ was generated by deleting of an 1.2 kb
Sph I fragment from pGAD425 (Han and Colicelli, 1995) and
religation of the vector.

1.4 Generation of a defined interaction library

To determine if the invention could be used in an improved
two-hybrid system for yeast, as shown in Figure 6 or Figure
7, a defined interaction library of plamids that express

various LexA and GAL4ad fusion proteins of interest was
constructed using the vectors and strains described in
sections 1.1 and 1.2. The orientation of the inserted
fragments was determined by restriction analysis and the
reading frame was checked by sequencing. The generated
constructs and the original plasmids described above are
listed in Table 2. The construction of pBTM117c-HD1.6, -HD3.6
and -SIM1 was described elsewhere (Wanker et al., 1997;
Probst et al., 1997). pBTM117c-HIP1 and pGAD427-HIP1 were
obtained by ligation of a 1.2 kb *Sal* I HIP1 fragment isolated
from pGAD-HIP1 (Wanker et al., 1997) into pBTM117c and
pGAD427, respectively. pBTM117c-MJD was created by inserting
a 1.1 kb *Sal* I/*Not* I MJD1 fragment (Kawagushi et al., 1994)
into pBTM117c, and pGAD427-14-3-3 was generated by inserting
a 1.0 kb EcoRI/NotI fragment of pGAD10-14-3-3 into pGAD427.
For the construction of pGAD427-HIPCT, a 0.5 kb *Eco* RI HIP1
fragment isolated from pGAD-HIPCT (Wanker et al., 1997) was
ligated into pGAD427. pGAD427-lexA and pGAD427-ARNT were
generated by insertion of a 1.2 kb *Sal* I/*Not* digested lexA
PCR fragment and a 1.4 kb *Sal* I/*Not* I ARNT fragment into
pGAD427 respectively.

It was shown that the fusion proteins LexA-SIM1 and GAL4ad-
ARNT specifically interact with each other in the yeast two-
hybrid system (Probst et al., 1997), because when both
hybrids were coexpressed in *Saccharomyces cerevisiae*
containing two integrated reporter constructs, the yeast *HIS3*
gene and the bacterial *lacZ* gene, which both contained
binding sites for the LexA protein in the promoter region,
the interaction between these two fusion proteins led to the
transcription of the reporter genes. The fusion proteins by
themselves were not able to activate transcription because
GAL4ad-ARNT lacks a DNA binding domain and LexA-SIM1 an
activation domain (Probst et al., 1997). In contrast it was
shown recently that the fusion proteins LexA-HIP1 and GAL4ad-
LexA are capable of activating the *HIS3* and *lacZ* reporter
genes without interacting with a specific GAL4ad or LexA

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fusion protein respectively. Thus, the yeast clones
expressing the LexA-HIP1 protein have to be designated as
false positives, because false positives are defined here as
clones where a GAL4ad fusion protein or a LexA fusion protein
alone without the respective partner protein activates the
transcription of the reporter genes without the need for any
interacting partner protein.

The predicted protein-protein interactions of these fusion
proteins are shown in Figure 8. It was shown that the fusion
proteins LexA-SIM1 & GAL4ad-ARNT, LexA-HD1.6 & GAL4ad-HIP1
and LexA-HD3.6 & GAL4ad-HIP1 specifically interact with each
other in the yeast two-hybrid system because they only
activate the reporter genes *HIS3* and *lacZ* when both proteins
are present in one cell (Probst et al. 1997; Wanker et al.
1997). In contrast, it was demonstrated that the LexA-HIP1
and GAL4ad-LexA fusion proteins are capable of activating the
reporter genes without the need for any interacting fusion
protein. The proteins LexA and GAL4ad and the fusion proteins
LexA-MJD and GAL4ad-14-3-3 which are also present in the
defined interaction library are unable to activate the
reporter genes either alone or when present in the same cell
with any other fusion proteins comprising the library.

Example 2:   Detection of clones expressing known
interacting proteins from false positives using the improved
two-hybrid system

Pairs of the yeast two-hybrid plasmids pBTM117cSIM1 &
pGAD427-ARNT, pBTM117c & pGAD427 and pBTM117c-HIP1 & pGAD427
were transformed into the yeast strain L40cc, and Trp+Leu+
transformants that contained at least one of each of the two
plasmids were selected on SD-leu-trp plates. Two
transformants from each transformation were investigated for
the presence of protein-protein interactions by testing the
ability of the yeast cells to grow on SD-leu-trp, SD-leu-
trp-his, SD-leu+CAN and SD-trp+CHX plates and by the ß-

galactosidase membrane assay (Breeden and Nasmyth, 1985).
Figure 9 shows that the yeast strains cells harboring both
the plasmids pBTM117c-SIM1 & GAD427-ARNT or pBTM117c-HIP1 &
pGAD427 grow on SD-leu-trp-his plates and turned blue after
incubation in X-Gal solution, indicating that the *HIS3* and
lacZ reporter genes are activated in these strains. In
comparison, the yeast strain harboring both the negative
control plasmids pBTM117c & pGAD427 was not able to grow on
this medium and also showed no lacZ activity. After selection
of the yeast strains harboring the different combinations of
the two-hybrid plasmids on SD-leu+CAN and SD-trp+CHX the
resulting strains were also analyzed by the ß-galactosidase
assay. After incubating the membrane containing all three
strains on SD-trp+CHX medium only progeny of the yeast strain
that originally harbored both the plasmids pBTM117c-HIP1 &
pGAD427 yet which had lost the pGAD427 plasmid through
counterselection turned blue after incubating in X-Gal
solution. This result indicates that this clone is a false
positive, because although showing a lacZ+ phenotype when
grown on SD-leu-trp-his medium, the LexA-HIP1 fusion protein
was also capable of activating the HIS3 and lacZ genes on SD-
trp+CAN medium without the need for any interacting fusion
protein. In comparison, the yeast strain harboring both the
plasmids pBTM117c-SIM1 & pGAD427-ARNT is a positive clone
that expresses interacting LexA and GAL4ad fusion proteins,
because both the LexA and the Gal4ad fusion proteins are
necessary for the activation of the reporter genes. If either
of the plasmids pBTM117c-SIM1 or pGAD427-ARNT is lost from
the strain by counterselection on SD-trp+CHX or SD-leu+CAN,
respectively, the resulting cells are no longer able to
activate the lacZ reporter gene and do not turn blue after
incubation in X-Gal solution. With the membranes from the SD-
leu+CAN plate false positive clones expressing an auto-
activating GAL4ad-LexA fusion protein were also detected by
the ß-galactosidase assay.

Example 3:   Generation of regular grid patterns of host
cells expressing potentially interacting fusion proteins

3.1 Generation of a regular grid pattern of clones from an
interaction library in microtiter plates using automation

To generate the well defined interaction library, the
constructs for the expression of the fusion proteins shown in
Figure 8 were pooled and 3 μg of the mixture was co-
transformed into yeast strain L40cc by the method of
Schiestel & Gietz (1989). The yeast cells co-transformed with
the constructs described in Table 2 were plated onto large 24
x 24 cm agar trays (Genetix, UK) containing minimal medium
lacking tryptophan leucine and histidine (SD-leu-trp-his).
The agar trays were poured using an agar-autoclave and pump
(Integra, Switzerland) to minimise tray-to-tray variation in
agar colour and depth. To maximise the efficiency of
automated picking, the transformation mixture were plated
such that between 200 and 2000 colonies per agar tray were
obtained after incubation at 30°C for 4 to 7 days.

Suitable changes to the hardware and software of a standard
picking robot designed for the picking of *E. coli* cells as
described by Lehrach et al. (1997) were made to account for
the specific requirements of yeast cells. The illumination of
agar-trays containing plated colonies was changed from the
dark-field sub-illumination to dark-field top-illumination to
differentiate yeast colonies from the lawn of non-transformed
cells. The existing vision guided motion system (Krishnaswamy
& Agapakis 1997) was modified to allow for a larger range of
`blob' size when selecting yeast colonies to pick from the
blob-feature-table returned by connectivity algorithms when
applied to a digital image of the agar tray containing
colonies. The clone inoculation routine was re-programmed to
ensure that cell material which had dried on the picking pins
during the picking routine was initially re-hydrated by 10
seconds of immersion in the wells of a microtiter plate

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before vigorous pin-motion within the well. This robotic
procedure ensured that sufficient cell material was
inoculated from each picking pin into an individual well of a
microtiter plate. The picking pins were sterilised after
inoculation to allow the picking cycle to be repeated by
programming the robot to brush the picking pins in a 0.3%
(v/v) solution of hydrogen peroxide, followed by a 70%
ethanol rinse from a second wash-bath and finally drying by
use of a heat-gun to evaporate any remaining ethanol from the
pins. Furthermore, an algorithm to automatically correct for
height variation in the agar was incorporated by referencing
the surface height of the agar in three corners and from
these points automatically estimating the surface plane of
the agar. The robot was further programmed to automatically
adjust both the imaging and picking heights according to the
agar surface height such that when a pin was extended into a
colony, it removed cells only from the top surface of the
colony and did not penetrate the whole colony into the growth
medium. Finally, we incorporated additional selection
criteria that would reliably sort between blue and white
colonies. Although the robot provided a method to select only
those `blobs' (colonies) within a range of average grey
scales (eg, > 80 for white colonies), this proved unreliable
since the actual value of average grey scale required to make
a correct discrimination varied across the agar-tray due to
slight variation in intensity of the illumination. Therefore,
a new method was implemented that automatically modified this
discrimination value based on the average illumination of a
region of the agar-tray as measured by the camera on a frame-
to-frame basis. Often, a `blue' colony that activated the
readout system was not uniformly blue across the its whole
area, but only the centre would be blue and the surrounding
cell material was white. In such cases, the connectivity
algorithms would detect two `blobs' - one (the blue centre)
lying directly on the other (the white surrounding) and
although the former would be ignored since it was blue, the
latter would be selected as its average grey-scale was

greater than the discrimination value. Such cases were
successfully selected against by ignoring any colonies that
had `holes' using a `number of holes' function of the image
analysis program, which flags those blobs which have a second
blob within their boundary.

Using these modifications to a laboratory picking robot,
individual yeast colonies were automatically picked from the
agar-trays into individual wells of a sterile 384-well
microtiter plate (Genetix, UK) containing sterile liquid
minimal medium lacking leucine and trptophan (SD-leu-trp) and
containing 7% (v/v) glycerol. The resulting microtiter plates
were incubated at 30°C for 36 hours, the settled colonies
were dispersed by vigorous mixing using a 384-well plastic
replicating tool (Genetix, UK) and then incubated for a
further 2 to 4 days. A picking success of over 90% wells
containing a growing yeast culture was achieved. After growth
of yeast strains within the microtiter plates, each plate was
labelled with a unique number and barcode. Each plate was
also replicated to create two additional copies using a
sterile 384-pin plastic replicator (Genetix, UK) to transfer
a small amount of cell material from each well into pre-
labelled 384-well microtiter plates and pre-filled with SD-
leu-trp-his/7% glycerol liquid medium. The replicated plates
were incubated at 30 °C for 3 days with a cell dispersal step
after 36 hours, subsequently frozen and stored at -70°C
together with the original picked microtiter plates of the
interaction library.

In this manner, a regular grid pattern of yeast cells
expressing potentially interacting yeast clones was generated
using a robotic and automated picking system. 384-well
microtiter plates have a well every 4.5 mm in a 16 by 24 well
arrangement. Therefore, for each 384-well microtiter plate a
regular grid pattern at a density greater that 4 clones per
square centimetre was automatically created.

3.2 Creation of regular grid patters of increased density

To generate arrays with higher densities, a computer-
controlled 96-well pipetting system (Opal-Jena) with
automatic plate-stacking, tip washing, liquid waste and
accurate x-y positioning of the microtiter plate currently
accessed by the tips was employed. The yeast two hybrid cells
that had settled in the bottom of the wells of the arrayed
interaction library as described above were re-suspended, and
a stack of these 384-well plates were placed into the input
stacker of the pipetting system. The system was programmed to
take a single 384-well microtiter plate containing the
arrayed yeast two-hybrid clones and parallel aspirate 10 μl
of culture medium and cells into each of the 96 pipette tips
from 96 wells of the 384-well plate. The inter-tip spacing of
the 96 tips was 9mm and the wells of the 384-well microtiter
plate were 4.5 mm so that cells were removed from only every
other well along each dimension of the 384-well plate. 8 μl
of the 96 aspirated samples contained in the tips were then
pipetted in parallel into one set of wells of a sterile 1536-
well microtiter plate (Greiner, Germany). Since the inter-
well spacing of this 1536-well microtiter plate is 2.25 mm,
yeast cells were deposited into only 1 every 4 wells along
each dimension of the 1536-well plate. The remaining 2 μl of
culture medium and cells was aspirated to waste before
sterilising each 96 tips in parallel. Sterilisation was
conducted by twice aspirating and washing to waste 50 μl of
0.3% (v/v) hydrogen peroxide stored in a first replenishable
wash-bath on the system, and then aspirating and washing to
waste 50 μl sterile distilled water stored in a second
replenishable wash-bath.

This plate-to-plate pipetting cycle was repeated 3 further
times, each time aspirating a different set of 96-clones from
the 384-well array of yeast 2-hybrid clones into a different
set of 96-wells in the 1536-well microtiter plate by moving
the microtiter plates relative to the 96-tips using the

accurate x-y positioning of the system. When all clones of the first 384-well microtiter plate had been sampled and arrayed into the 1536-well plate, the first 384-well microtiter plate was automatically exchanged for the next 384-well microtiter plate, and the yeast 2-hybrid clones arrayed in this second 384-well plate were similarly arrayed into the 1536-well plate. When the yeast 2-hybrid clones contained within four 384-well microtiter plates had been automatically arrayed in the first 1536-well plate, filling all wells, the 1536-well plate was automatically exchanged for a second sterile 1536-well plate stored in the second stacking unit of the pipetting system. The whole process was repeated until all yeast 2-hybrid clones of the interaction library had been automatically transferred form 384-well to 1536-well microtiter plates.

In this manner, a regular grid pattern of yeast cells expressing potentially interacting yeast clones using a computer-controlled pipetting system was generated. 1536-well microtiter plates have a well every 2.25 mm in a 32 by 48 well arrangement. Therefore, for each 1536-well microtiter plate we automatically created a regular grid pattern at a density greater than 19 clones per square centimetre.

3.3 Generation of a regular grid pattern of clones from an interaction library on porous carriers using automation

A high-throughput spotting robot such as that described by Lehrach *et al.* (1997) was used to construct porous planar carriers with a high-density regular grid-pattern of yeast clones from the defined interaction library contained within 384-well microtiter plates. The robot recorded the position of individual clones in the high-density grid-pattern by the use of a pre-defined duplicate spotting pattern and the barcode of the microtiter plate. Individually numbered membrane sheets sized 222 x 80 mm (Hybond N+, Amersham UK) were pre-soaked in SD-leu-trp-his medium, carefully laid on a

sheet of 3mm filter paper (Whatmann) pre-soaked in the same
medium and placed in the bed of the robot. The interaction
library was automatically arrayed as replica copies onto the
membranes using a 384-pin spotting tool affixed to the robot.
Five different microtiter plates from the first copy of the
interaction library were replica spotted in a `3x3 duplicate'
pattern around a central ink guide-spot onto 10 nylon
membranes - corresponding to approximately 1900 clones
spotted at a density of approximately 40 spots per cm^2. On
each replica membrane three different control clones were
spotted, each from a microtiter plate that contained the same
control clone in every well. One control clone expressed the
fusion proteins LexA-SIM1 & GAL4ad-ARNT, a second control
clone the fusion protein LexA-HIP1, while a third expressed
fusion protein GAL4ad-LexA, and all were spotted in order to
test the selection, counterselection and the ß-gal assay
features of the method. To ensure the number of yeast cells
on each spot was sufficient for those membranes which were to
be placed on the counterselection media plates, the robot was
programmed to spot onto each spot position 5 times from a
slightly different position within the wells of the
microtiter plates. The robot created a data-file in which the
spotting pattern produced and the barcode that had been
automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of
solid agar media in 24 x 24 cm agar-trays. Six membranes were
transferred to SD-leu-trp-his media and two each of the
remaining membranes were transferred to either SD-trp+CHX or
SD-leu+CAN media. The yeast colonies were allowed to grow on
the surface of the membrane by incubation at 30 °C for 3
days.

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3.4   Generation of a regular grid pattern of clones from an
interaction library on non-porous carriers using automation

The plasmid pGNG1 (MoBiTec, Germany) carries a green
fluorescent protein variant under the control of a LexA
operator. This variant, GFPuv, is up to 16 times brighter
that the wild-type variant isolated from *Aequora victoria*
(Ausubel et al., 1995; Short protocols in molecular biology,
3rd ed. John Wiley & Sons, New York, NY.). The yeast 2um
origin of replication and the auxotrophic marker URA3
maintains the plasmid in ura3 mutant yeast strains. This
plasmid should act as a readout system to detect single
fusion proteins or interacting fusion proteins able to
activate the readout system in the method of invention
described herein. As is known in the art, green fluorescent
proteins and its variants are considered suitable reporter
genes in most host-cell types. Therefore, it would be
possible for a person skilled in the art to incorporate this
gene within other host-cell types and interaction systems as
disclosed in this invention.

The yeast strain L40ccu was transformed with the plasmid
pGNG1 (MoBiTec, Germany) using the method of Schistel & Gietz
(1989), and a resulting stable transformant clone cultured in
minimal medium lacking uracil and subsequently used to
generate two further yeast clones, each containing two
genetic elements. The first strain, GNGp, was generated by
co-transformation of a mixture of the plasmids pBTM117c-SIM1
and pGAD427-ARNT co-transformed into L40ccu already carrying
the reporter plasmid pGNG1. The second strain, GNGn, was
generated by co-transformation of a mixture of the plasmids
pBTM117c-MJD and pGAD427-14-3-3 co-transformed into L40ccu
already carrying the reporter plasmid pGNG1. In both cases,
the transformations were conducted using the method of
Schistel & Gietz (1989), and transformants were selected by
plating on minimal media lacking uracil, trptophan and
leucine.

Individual colonies from the two transformations were picked
into individual wells of 384-well microtiter plates as
described in section 3.1 except that the microtiter plates
contained liquid minimal medium lacking uracil, tryptophan
and leucine. One microtiter plate was created that contained
individual colonies of the GNGp yeast strain, and another
carrying colonies of GNGn. Using a spotting robot (Lehrach et
al., 1997) fitted with high precision spotting tool carrying
16 pins in a 4 x 4 pattern, the clones were arrayed onto
poly-lysine coated glass-slide (Sigma, US). The clones were
spotted at a spacing of 440 um, with a spot diameter of
approximately 300 um generating a density of over 490 clones
per square centimetre. To increase the amount of cell
material deposited at each spot, the robot was programmed to
spot onto each spot position 10 times from a slightly
different position within the wells of the microtiter plates.
It is well known in the art that piezo-ink-jet micropipetting
systems (Kietzmann et al., 1997, Schober et al., 1993) can
create regular grid pattern of clones at an even greater
density. Indeed, grid densities of over 1600 spots per quare
centimeter have been achieved with such systems.

The fluorescent readout system of cells in the regular grid
pattern of cells was then visualised using a sensitive CCD
camera (LAS1000, Fuji, Japan). Appropriate excitation light
was provided and an emission filter appropriate for the
emission spectrum of GFP_{uv} was fitted to the lens. Other
imaging systems could be utilised to investigate the regular
grid pattern of clones. For example, laser-scanning systems
including laser scanning confocal microscopes would be
preferred when imaging very high density regular grid
patterns, or for those formed from a small number of host
cells deposited at each position.

It was shown that the fusion proteins LexA-SIM1 and GAL4ad-
ARNT can interact and activate a readout system under control

of the LexA operator. Since the GNG$_{uv}$ reporter gene is under
the control of a LexA operator, a cell carrying the pGNG1
plasmid and expressing these fusion proteins should fluoresce
under UV light. In contrast, the fusion proteins LexA-MJD and
GAL4-14-3-3 were shown unable to activate the same readout
system. Image analysis of the digital image of the regular
grid pattern of yeast cells, demonstrated that indeed, the
GNGp yeast strain did fluoresce while the GNGn did not.

As an alternative to pGNG1 a person skilled in the art could
subclone an improved GFP mutant as described in Anderson et
al. (1996). Replacement of the URA coding sequence in pLUA
(section) with GFP is performed by using appropriate anchor
primer to amplify the GFP mutant. Using the appropriate
growth media the analysis can be performed as described
above.

Example 4:   Detection of the readout system in a regular
grid pattern.

4.1 Detection of readout system activation in a regular grid
pattern of clones from an interaction library on planar
carriers using digital image capture, processing and
analysis

Two membranes from each of the selective media described in
section 3.3 were assayed for lacZ expression using the ß-gal
assay as described by Breeden & Nasmyth (1985) and air dried
overnight. For each membrane, a 24-bit digital BMP (bitmap)
image of the ß-gal assay was captured using a standard A3
computer scanner, and the images were stored on computer. The
yeast strain used to create the defined interaction library
was an *ade2* auxotrophic mutant, and those colonies that grew
yet did not activate the readout system were pink in colour
when mature. Since image analysis programs used for the
analysis of DNA grids use single channel (grey-scale) images,
it was necessary to convert this colour image to an 8-bit

grey-scale image. However, the pink colour of colonies not
expressing the ß-gal reporter gene, when converted to grey-
scale, would lower the contrast between positive and negative
activation states of the readout system. Therefore, the pink-
red colours of the image were re-mapped to light yellow
before processing the remapped 24-bit colour image to a
colour-inverted 8-bit grey-scale TIF (tagged image file
format) using the software Photo Magic (Micrografix, USA).
One non-inverted 8-bit grey-scale image of the defined
interaction library that was grown on membranes placed on
each of the 3 selective media and subsequently assayed for β-
gal activity is shown in Figure 10.

Individual clones of the interaction library can be
identified and their position on the high-density spotted
filter converted to specific wells in the microtiter plates
using an automated image analysis system as described by
Lehrach et al. (1997). Here, the basic grid and node position
of each clone is determined through an iterative sampling
scheme proposed by Geman & Geman (1984). Once the node
positions have been determined, the average grey-scale value
of a pixel mask appropriately sized for the average colony
diameter is recorded from the image for every colony on the
filter. From these intensity data, global and block-specific
background corrections are made, giving greater weight to the
local block-specific background. Each colony is then
classified into one of four ß-galactosidase activities by
appropriate binning values of the background-corrected
intensities.

Positive clones that expressed interacting fusion proteins
were detected from false positive clones by considering the
activity of ß-galactosidase of clones grown on spotted
membranes laid on the various selective media. Positive
clones should activate the lacZ reporter gene on SD-leu-trp-
his media and turn blue on incubation with X-Gal solution,
but not on either of the two counterselective media. False

positive clones should activate the reporter gene and turn
blue on incubation with X-Gal solution on at least one
counterselective media as well as on the SD-leu-trp-his
medium.

Figure 11 shows magnified images of a ß-gal assay of clones
grown on the membranes which had been placed on the three
selective media. Within the magnified region of the membranes
shown in Figure 11a, two clones were detected as positive
clones that express interacting fusion proteins since they
activated the lacZ reporter gene on SD-leu-trp-his media, but
not on either of the two counterselective media, and whose
spotted positions are circled. The two clones were identified
by their microtiter plate address within the interaction
library as 06L22 and 08N24 respectively. All other clones
spotted within this region of the membrane were detected as
false positive since they express ß-galactosidase on SD-
trp+CHX medium as well as on SD-leu-trp-his medium.

Expression of the LacZ reporter gene for the three control
clones spotted onto the same membranes confirm these results.
The positive control clone that expresses the interacting
fusion proteins LexA-SIM1 & GAL4ad-ARNT should show a LacZ+
phenotype when grown on SD-leu-trp-his medium, but LacZ- when
grown on either of the counterselective media. This control
clone was spotted at position 03 in the region of the
membranes shown in Figure 11b, of which one example is
circled. The pattern of ß-gal activity for this positive
control clone on the three selective media is as predicted.
The false positive control clone that expresses the fusion
protein LexA-HIP1 and the false positive clone that expresses
the fusion protein GAL4ad-LexA are spotted at positions 02
and 01 respectively. Both false positive control clones show
a LacZ+ phenotype when grown on SD-leu-trp-his media, but are
detected as false positive clones by the method of the
invention since they also show a LacZ+ phenotype on SD-
leu+CAN or SD-trp+CHX media, respectively. The clones spotted

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at position 04 are from the defined interaction library, and
from their LacZ+ phenotype when grown on SD-leu+CAN media are
predicted to be false positive clones.

The image analysis system described above was used to
automatically identify those individual clones on each high-
density regular grid pattern that had activated the LacZ
readout system. This was conducted for each of the membranes
grown on the three selective media, and the intensity of ß-
galactosidase activity for each clone grown on the three
media was automatically recorded by the program using a scale
from 0 to 3 (no activity, weak activity, medium activity,
high activity). These data for all clones on a given membrane
were saved in a computer file, and the ß-galactosidase
activity for a given clone was related to its activity when
grown on the other two selective media using a computer
program. This program was used to query and identify all
clones from the interaction library that had activated the
reporter gene when grown on SD-leu-trp-his (score greater
than 0), yet had not on either of the counterselective media
(score on both media equal to 0). Figure 12a shows a subset
of these clones identified using this data-query procedure,
and Figure 12b shows that the two clones 06L22 and 08N24 are
found within this automatically identified data-set of
positive clones.

4.2　Detection of readout system activation in a regular grid
pattern of clones from an interaction library in microtiter
plates using digital image capture, processing and analysis.

The interaction library comprising the yeast cells as
described in section 3.1 were screened in microtiter plate
format to identify those cells that express interacting
fusion proteins. First, microtiter plates containing the
interaction library were removed from frozen storage and
thawed to room temperature. Second, each plate was replicated
and labelled as described in section 3.1 to create additional

copies for screening, each into 3 separate selective media.
Cells were transferred into 384-well microtiter plates pre-
filled with 40 ul of the liquid selective media SD-leu-trp,
SD-leu+Can or SD-trp+CHX. Third, after growth for 4 days at
30°C, 10 ul of Yeast One Step Yeast Lysis Buffer containing
Galacton-Star and Sapphire II (Tropix, US) was added, the
cells were dispersed using a plastic replication tool, and
the plates incubated for 40 min at 37°C. Finally, a digital
image of six plates was obtained in parallel using a LAS1000
CCD camera (Fuji, Japan), by placing the plates side-by-side
in a two by three arrangement. The ß-galactisidase substrate,
Galacton-Star in combination with Sapphire II (Tropix, US)
generates detectable luminescent light on activation of the
ß-gal reporter gene in the yeast-cells, and an exposure time
of 5 minutes was used to collect sufficient signal. The grey-
scale digital images were captured, saved on computer and
subsequently analysed using the image analysis system
described in section 4.1. However, in this case, the position
of each clone was far simpler to determine due to the lower
density of the regular grid pattern of clones in the
microtiter plate. Second, the size of the pixel mask used to
measure the average pixel intensity was approximately that of
the size of the microtiter plate well. Positive clones in the
six microtiter plates were identified by image analysis of
the digital images from clones grown in the three selective
media, and these data processed by the computer program as
described in section 4.1.

Example 5:   Identification of individual members of the
interaction

The interaction library constructed for this example was
composed of known fusion proteins with predicted interactions
as shown in Figure 8. A real positive clone from this defined
interaction library is therefore expected to express the
interacting fusion protein-pairs LexA-SIM1 & GAL4ad-ARNT,
LexA-HD1.6 & GAL4ad-HIP1 or LexA-HD3.6 & GAL4ad-HIP1 and

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hence contain the corresponding pairs of plasmid constructs
pBTM117c-SIM1 & pGAD427-ARNT, pBTM117c-HD1.6 & pGAD427-HIP1
or pBTM117c-HD3.6 & pGAD427-HIP1, respectively. The
identification of individual members that comprise an
interaction between fusion proteins that are expressed within
a single cell can be made by a variety of means as outlined
in Figure 1, Figure 6 and Figure 7. Three independent
methods, nucleic acid hybridisation, PCR and DNA sequencing
were used to identify the individual plasmid constructs that
expressed the interacting fusion proteins in the positive
clones 06L22 and 08N24.

5.1   Identification of individual members of the interaction
by nucleic acid hybridisation

The four membranes which had been placed on the SD-leu-trp-
his medium and had not been used to assay ß-gal activity were
processed according to the procedure described in Larin &
Lehrach (1990) in order to affix the DNA contained within the
clones of the interaction library onto the surface of the
membrane. A 1.1 kb DNA fragment of SIM1 and a 1.3 kb DNA
fragment of ARNT were radioactively labeled by standard
random priming procedures for use as a hybridisation probe
(Feinberg & Vogelstein, 1983). Each probe was heat denatured
for 10 min at 95 °C and hybridised overnight at 65 °C in 15
ml of 5% SDS/0.5M sodium phosphate (pH 7.2)/1 mM EDTA with a
high-density spotted membrane with DNA from the interaction
library affixed to it as prepared above. The membranes were
washed once in 40mM sodium phosphate/0.1%SDS for 20 min at
room temperature and once for 20 min at 65 °C before wrapping
each membrane in Saran wrap and exposing it overnight to a
phosphor-storage screens (Molecular Dynamics, USA). A digital
image of each hybridised membrane was obtained by scanning
the phosphor-storage screen using a phosphor-imager
(Molecular Dynamics, USA). The digital image was stored on
computer and was analyzed using the image analysis system for
the analysis of DNA arrays as described in Lehrach et al.,

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1997 which marked positive hybridisation signals with square
blocks. Figure 13 shows a magnified region of each hybridised
membrane corresponding to that shown in Figure 11a containing
the clones 06L22 and 08N24, the spotting position of which
are circled. These clones were predicted to express either
the interacting fusion protein-pairs LexA-SIM1 & GAL4adARNT,
LexA-HD1.6 & GAL4ad-HIP1 or LexA-HD3.6 & GAL4ad-HIP1, and
hybridisation with the specific SIM1 and ARNT probes have
shown that both clones contain the plasmid constructs
pBTN117c-SIM1 and pGAD427-ARNT.

5.2   Identification of the individual members of the
interaction by nucleic acid amplification and sequencing

The individual clone 06L22 was recovered from the frozen
plates of the original interaction library and inoculated
into SD-leu-trp-his liquid medium. This culture was allowed
to grow for 3 days at 30 °C and the corresponding plasmids
contained in the clone were isolated using a QiaPrep (Qiagen,
Hilden) procedure. Duplex PCR was used to simultaneously
amplify the inserts contained within the plasmid constructs
using primer-pairs specific for either the pBTM117 or pGAD427
plasmids. The presence of the SIM1 and ARNT inserts was
confirmed for clone 06L22 by electrophoresis of the amplified
PCR products against separate control amplifications of the
inserts from plasmids pBTM117c-SIM1 and pGAD427-ARNT as size
markers (Figure 14).

PCR of the individual inserts from individual plasmids
carried by clone 06L22 was conducted as above except by using
only the respective primer pair for the required plasmid. The
individual inserts were also amplified directly from the
yeast culture using a Whole Cell Yeast PCR Kit (Bio 101,
USA). The pairs of inserts isolated from clone 06L22 either
by amplification from the extracted plasmid DNA or by direct
PCR of the yeast clone were subjected to DNA sequencing by
standard protocols.

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The 1.26Kb inserts amplified using the primers specific to
plasmid pBTM117 were confirmed as the expected fragment of
the SIM1 gene by comparison of the known sequence for this
gene (Probst et al., 1997). Likewise, the 1.37Kb inserts
amplified using the primers specific to the pGAD427 plasmid
were confirmed as the expected fragment of the ARNT gene.

Example 6:   Detection and identification of interacting
proteins using a large-scale and automated application of the
improved 2-hybrid system

A scheme utilizing the method of the invention within a
large-scale and automated approach for the parallel detection
of clones that express interacting fusion proteins and the
identification of members comprising the interactions is
shown in Figure 6. Yeast clones from an `interaction library'
that express interacting proteins are identified on a large-
scale by the use of visual inspection or digital image
processing and analysis of high-density gridded membranes on
which their ß-galactosidase activity has been assayed after
growth on various selective media. Automated methods as
described in earlier examples are used to effect the
production of the interaction library and high-density
spotted membranes, and the analysis of digital images of the
ß-gal assay and hybridisation images.

6.1 Generation of an interaction library for a higher
Eukaryote

A random-primed and size selected (1 - 1.5Kb) cDNA library of
40-hour post fertilisation Sea Urchin embryos
(*Strongylocentrotus purpuratus*) cloned into the *Not* 1/*Sal* 1
sites of pSport1 by standard procedures (Life Technologies,
USA) was obtained as a gift from A.Poustka. 100 ng of this
library, representing the estimated 6000 different
transcripts expressed at this developmental stage (Davidson,
1986), was transformed into electro-competent *E.coli* cells by

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standard electroporation techniques. Recombinant clones were
selected by plating the transformation mixture on 2xYT/100
μg/ml amplicillin contained in 24 x 24 cm agar-trays
(Genetix, UK). After growth for 18 hours at 37 °C, the
resulting recombinant colonies (estimated to be 20,000 per
tray) were washed from the 5 trays using 50 ml of LB liquid
media for each tray. The amplified cDNA library cloned into
pSport was isolated from this wash mixture by a QiaPrep
(Qiagen, Germany) plasmid extraction procedure. Approximately
1 μg of the library inserts were then isolated from the
plasmid DNA by *Not* 1/*Sal* 1 digestion and size selected (1
1.5Kb) by agarose gel purification using standard procedures.

Two pools representing all three reading frames of the two
vector series pGAD428 and pBTM118 were prepared by *Not* 1/*Sal*
1 digestion and pooling of 1 μg each of vectors pGAD428 a, b
& c and pBTM118 a, b & c respectively. The insert mixture
that was isolated as above was split into two equal fractions
and 300 ng was ligated with 50 ng of each prepared vector-
series pool. Following ligation, each reaction was then
separately transformed into electro-competent *E.coli* cells,
and recombinant clones for each library were selected on five
24 x 24 cm plates using kanamycin or ampicillin for the
pGAD428 or pBTM118 libraries respectively. Approximately 500
μg of the pBTM118 and 500 μg of the pGAD428 libraries was
extracted from the two sets of *E.coli* transformants by
washing off the plated cells and a subsequent QiaPrep plasmid
extraction of the wash mixture as described above.

To generate the interaction library, molar-equivalent amounts
of the DNA binding and activation domain libraries were
pooled, and 20 μg of this mixture was co-transformed into the
yeast strain L40cc by the method of Gietz et al. (1992). The
resulting transformation mix was plated on a single 24 x 24
cm agar tray. The agar-trays were prepared as described in
section 1.3.1. A total of twenty transformations were
prepared and plated onto separate agar trays yielding an

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average of 1500 yeast colonies per tray after 7 days of
incubation at 30 °C.

6.2   Creation of a regular grid-pattern of an interaction
library in microtiter plates

To create a regular grid-pattern of the interaction library,
the agar-trays containing yeast colonies were placed in the
modified laboratory picking robot and individual clones were
automatically picked as described in section 3.1. A total of
30 384-well microtiter plates were generated and represented
an interaction library of greater than 10,000 clones for the
study organism. After growth of yeast clones in the wells of
the microtiter plate, the library was replicated to generate
3 further copies, labelled and all copies were stored at ⅜
70°C to provide for analysis at a later date as described in
section 3.1.

6.3   Creation of a regular grid-pattern of an interaction
library on planar carriers

To provide for efficient analysis of the interaction library,
the clones comprising it were arrayed at high density on 222
x 222 mm porous membranes (Hybond N+, Amersham, UK) using the
method described in section 3.3. A total of twenty replica
membranes, each arrayed in a `3 x 3 duplicate' regular grid-
pattern of clones using 23 384-well microtiter plates from a
thawed copy of the stored interaction library were produced.
On each replica membrane, one microtiter plate was
aditionally arrayed in position 24 containing 8 different
control clones representing known positive, negative and
false positive clones.. This pattern corresponded to over
9000 yeast two-hybrid clones spotted at a density of
approximately 40 clones cm^{-2}. To ensure the number of yeast
cells on each spot was sufficient for the four membranes
which were to be placed on the counterselection media plates,
the robot was programmed to spot onto each spot position 5

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times from a slightly different position within the wells of
the microtiter plates. The robot created a data-file in which
the spotting pattern produced and the barcode that had been
automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of
solid agar media in 24 x 24 cm agar-trays. Fourteen membranes
were transferred to SD-leu-trp-his media and three each of
the membranes which had been spotted five times were
transferred to either SD-trp+CHX or SD-leu+CAN media. The
yeast colonies were allowed to grow on the surface of the
membrane by incubation at 30 °C for 3 days.

6.4   Detection of the readout system in a regular grid
pattern and analysis using digital image analysis to identify
positive clones

To provide for the efficient identification of individual
clones that expressed interacting fusion proteins, the
activation state of the individual clones grown on the porous
carriers was examined in a highly parallel manner. The
replica arrays of the interaction library grown on the six
membranes placed on the counterselective media, plus three
further membranes which were placed on SD-leu-trp-his medium
as described above, were assayed for lacZ activity, a digital
image of each was captured and image-processed as described
in section 1.4.1. Figure 15 shows an grey-scale image of
readout system activation for individual clones from the
interaction library arrayed in a regular grid-pattern on a
membrane filter and grown on SD-leu-trp-his medium.

The activation state of the readout system for each
individual clone in the regular grid-pattern grown on the
three selective media was recorded from each digital image
using the image analysis system described in section 4.1.
These data were collected for the interaction library grown
on three replica-membranes for each of the selective media

SD-leu-trp-his, SD-leu+CAN & SD-trp+CHX, and was related
together for each individual clone using the computer program
shown in Figure 12a.

This program was used to query these data and identify those
clones that had activated the readout system when grown on
two out of three SD-leu-trp-his replica membranes, but not
when grown on any of the two sets of three replica membranes
placed on the two counterselective media SD-leu+CAN or SD-
trp+CHX. The data-base correctly identified the eight
different control clones each arrayed in 48 wells of the 24th
microtiter plate. A total of 7539 clones from the interaction
library arrayed in 23 384-well microtiter plates were thus
identified as positive clones - clones that only activated
the readout system when both plasmids (and hence fusion
proteins) were expressed in the cell. 3983 clones were
identified as false-positive clones as they also activated
the readout system when grown on SD-trp+CHX medium ⁑ the
growth medium that eliminated the plasmid expressing the
activation domain fusion protein. 113 clones were identified
as false positive clones by activating the readout system
when grown on SD-leu+CAN medium ⁑ the growth medium that
eliminated the plasmid expressing the DNA binding fusion
protein. These data were automatically made available to a
table of the relational database holding information on each
clone of the interaction library as described in Example 7.

This relatively high number of false-positive clones
identified following SD-trp+CHX selection can be explained
since on elimination of the activation domain plasmid, the
DNA-binding domain fusion protein is tested for its ability
to activate the readout system without any partner protein.
It is known that many transcripts expressed in early Sea
Urchin embryos are transcription factors, and that fragments
of transcription factors can commonly cause false positives
in the yeast two-hybrid system when expressed as the DNA-
binding domain fusion protein. Therefore, these results

demonstrate that the above method can efficiently eliminate
large-numbers of false positive clones from a large-scale
library vs. library screen interaction screen.
6.5 Identification of the individual members of the
interaction by nucleic acid amplification and sequencing

A total of 96 positive clones were randomly selected from the
database and recovered from a frozen copy of the interaction
library clones stored in 384-well microtiter plates. The DNA
sequences cloned into the pGAD428 and pBTM118 vectors carried
by each clone were directly amplified as described in section
5.2 except that the direct PCR reactions were conducted in
96-well microtiter plates using a high-thoughput water-bath
thermocycling machine (Maier et al., 1994).

Standard sequencing approaches were employed to characterise
the nucleic acids encoding the DNA-binding domain fusion
proteins of the positive clones following pBTM428- specific
96-well PCR as described above. Similarly, the sequence of
the insert encoding for the activation-domain fusion protein
following pGAD118-specific PCR was determined. Sequence
comparison of these insets against published DNA sequences
using standard sequence comparison tools (e.g. BAST),
identified that one interaction involved two previously
unidentified gene fragments that were expressed by the
positive-clone located in plate 5, well K20. From the
predicted protein sequence these two genes were designated
Protein A and Protein B.

6.6 Identification of individual members of the interaction
by nucleic acid hybridisation

Regular grid patterns of the nucleic acids encoding the
fusion proteins from the interaction library were
constructed. The membranes which had been placed on the SD-
leu-trp-his medium and had not been used to assay ß-gal
activity were processed according to the procedure described

in Larin & Lehrach (1990) in order to affix the DNA contained within the clones of the interaction library onto the surface of the membrane. The DNA fragment that encoded Protein A isolated as above, was radioactively labelled by the method of Feinberg & Vogelstein (1983). This labelled probe was hybridised to an array with DNA from the interaction library affixed to it, and the array washed and detected as 5.1.

The number and identity of hybridisation-positive clones was determined for each hybridisation using the automated image analysis system described in Lehrach et al., (1997). Seven clones from the interaction library were identified as hybridisation-positive for the probe encoding Protein A. Figure 16 shows a digital image of a DNA array hybridised with the gene fragment encoding Protein A with the hybridisation-positive clones identified and marked by the automated image analysis system, and Figure 17 represents a graphical representation of the positives found by this analysis. The database described in Example 7 was used to refer to the list of clones generated by the image analysis program and identify those hybridisation-positive clones that were interaction-positive clones and hence eliminate any false positive clones from further analysis. As expected, a hybridisation-positive clone was the clone 5K20 from which the probe corresponding to Protein A was obtained.

To extend the interaction pathway from Protein A, a second filter was hybridised with a radioactive labelled probe generated from the fragment coding for Protein B. Analysis of the hybridisation signals with the database described in Example 7 resulted in the identification of eight interaction-positive clones that carried the gene fragment encoding for Protein B. Figure 18 shows a graphical representation of the hybridisation-positive and interaction-positive clones identified with probe B (open circles) and probe A (red circles). Two clones (5K20 and 3L11 marked by "A/B") gave a hybridisation signal with both probe A and

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Probe B, indicating that both these positive clones expressed
the same interacting fusion proteins.

To further extend the interaction pathways of proteins A and
B, the DNA binding and activation domain plasmids were
extracted from one interaction-positive clone that gave a
hybridisation signal only with probe B (clone 6D18). DNA
sequencing of the inserts carried by these genetic elements
confirmed the presence of a gene fragment encoding for
Protein B in the DNA binding domain plasmid. Sequence
analysis showed that the activation domain plasmid carried a
fragment for another unknown gene coding for Protein C. This
gene fragment was used as a probe to another array and the
data analysed as above. Figure 19 shows the results of this
hybridisation (marked with diamonds), together with that from
the previous two hybridisations. A total of six interaction-
positive clones were identified as carrying genetic elements
encoding for Protein C. Three of these interaction-positive
clones were previously shown to hybridise with probe B (4G19;
1D7; 6D18) and two clones to hybridise with probe A (1C22;
3A11). A graphical view of the interactions identified by
these three simple hybridisations is outlined in Figure 19.
Question marks represent possible further steps in the
network which could be further investigated by a similar
investigation of the genetic elements carried by the
remaining hybidisation-positive clones for probes A, B or C.
Indeed, by following this focused hybridisation approach, 14
different protein-protein interactions were identified by a
total of nine hybridisations and subsequent sequencing of the
inserts encoding the interacting members. All these data were
enteredinto the data-base described in Example 7.

6.7 Automated rearraying of positive clones

The 3443 positive clones identified as described above were
distributed across all 23 microtiter plates of the
interaction library. To greatly facilitate further analysis

of positive clones, it was advantageous to individually
physically isolate clones and to generate a second, re-
arrayed regular grid-pattern of positive clones, preferably
within a further set of 384-well plates.

Existing rearraying robots such as described by Stanton et
al, (1996), Lehrach et al., (1997) or those sold by
commercial sources (Genetix, UK) system failed to provide a
satisfactory inoculate when transferring yeast cells from
individual wells of a source (`mother') 384-well plate
containing the original interaction library in wells of a
new, sterile 384-well destination (`daughter') plate
containing growth medium. Therefore, the existing transfer
pins were replaced by straight 2 mm diameter pins that
terminated in a flat end. Secondly, the inoculation procedure
was modified to maximise the amount of dried cell material
carried on the pin that was transferred into the new well
within the daughter plate as described for automated picking
of yeast colonies in section 3.1. The pins were sterilised
between rearraying cycles by a 0.3% hydrogen peroxide wash-
bath, 70% ethanol wash-bath and heat-drying procedure as
described in section 3.1.

The list of positive clones, together with their plate-well
location was generated from the data-base described in
Example 7 and automatically loaded as a computer file onto
the rearraying robot. The robot automatically took the mother
plate containing the first positive yeast two-hybrid clone by
reference to the data file and read and recorded the barcode
of the plate. Individual and sequential pins of the 96-pin
rearraying head were positioned above and lowered into the
required wells from this first plate, and the mother plate
was automatically exchanged when all positive clones had been
sampled. When all 96-pins had been used to collect inoculates
of positive clones, the head was automatically moved over to
the first 384-well daughter plate containing SD-leu-
trp/7%glycerol and inoculated all 96-pins in the first set of

wells as described above. A data output file was then updated
which related the new plate-well location of a given positive
clone in the re-arrayed library to its old plate-well
location in the original interaction library. All pins were
then sterilised as described, and the cycle completed until
all positives clones had been transferred from the
interaction library to a new plate-well location comprising
the re-arrayed library. The data output file was then
transferred to the central computer database to append a
table in the data-base described in Example 7 to record the
correct location of a given positive clone in the re-arrayed
interaction library. The resulting clones in the daughter
plates were replicated into two further copies and stored at
-70 °C as described in section 3.1.

Example 7: Generation of a data-base of interactions.

Central to the scheme (Figure 2) is a data-table holding
relevant information on each member of an interaction - the
cDNA-Table - where a separate record in the table represents
each member of an interaction, and members are indicated to
form interactions by sharing the same clone name. It is
advantageous to structure the core data-table in this way for
several reasons. First, the same core table can be used to
hold data on cDNAs from different kinds of genetic libraries
(for example, standard cDNA or genomic libraries) which can
be generated during a global analysis using various genomic
techniques, not just interaction data. Secondly, each of the
members of an interaction, or genetic fragments may be
further characterised by a number of ways for different sets
of data. Of direct relevance to protein-protein interaction
for a given genetic fragment in the cDNA_Table is first, the
Gene_Table, which provides a direct relationship to the
fragment's DNA sequence, nucleotide homology match (for
example through BLAST searching) and the corresponding gene
name. Second, the Domain_Table provides facility to directly
access data of the fragment's in-frame translation, amino

acid homology match (for example through BLASTN searching)
and any 2 or 3-dimensional structural information which may
be known or can be predicted. As is commonly known in
molecular biology, there are many ways in which a given
genetic fragment may be characterised, and this data-base
structure provides the facility to relate from the central
cDNA_Table to any other table holding data describing said
characterisation as may be appropriate. For example, those
holding data on genetic, expression, target validation,
protein biochemistry or library construction information. Of
particular relevance to the method of invention, is the
relationship of a given cDNA fragment to a table holding
information on oligofingerpriting data. Said
oligofingerpriting data can be used to identify each member
of an interaction in a highly parallel manner and includes
fields for data such as cluster number, confidence of cluster
membership and predicted gene homology for that cluster
(Maire et al., 1994). Third, such a data-base structure will
more easily enable tertiary or higher order interactions to
be incorporated within the same data table. This is in
contrast to a structure in which interactions rather than
members of an interaction were the basic object or record in
a data table, and for each higher order interaction a new
data-table would be needed or an existing data-table
modified.

In the case of a yeast two-hybrid interaction screen one
related table would be the Y2H_Table. Said table may include
information for a given clone pertaining to cloning and
experimental details of its creation, the tissue and library
from which it was derived, its physical location to enable
easy access for further studies, whether it was derived from
the mating of given Mata and Matα strains. Importantly, the
Y2H_Table holds information pertaining to the interaction
class of the clone ⚥ where said interaction class is defined
as whether the clone was a positive clone, negative clone, or
a false positive with respect to either the activation domain

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(AD) or biding domain (BD) fusion protein. The value for said
interaction class is easily derived for a large number of
clones by the method of invention described in earlier
examples.

To assist any focused approach to identifying members
comprising the interactions, the Hyb Table is provided. This
table relates for a given clone, the hybridisation intensity
obtained with a given probe in a hybridisation experiment
using a given high density array. Said high-density array to
be related to tables holding data from the spotting robot
such as the defined spotting pattern used, the method by
which the array was produced and the identity of the library
and clones arrayed on said array. The incorporation of these
tables within a user interface will enable this embodiment of
the method of invention to be easily conducted by displaying
to the user the physical location of a given positive yeast
two hybrid clone that hybridised to a given probe. Said two-
hybrid clone can then be recovered, the members comprising
the interaction isolated by PCR and sequenced. Said sequenced
members of an interaction then provide data to be entered
into the cDNA_Table and other related tables on further
analysis. Said member to then be used as a second
hybridisation probe onto an array to identify the next step
in an interacting pathway by the same procedure.

On collection of a substantial number of interacting members
within the cDNA_Table, these data can be curated by manual
and/or expert systems to update a definitive data table for
example the PathCode_Table. Said definitive database to hold
the highest quality information on interactions from the
cDNA_Table, where said highest quality information on
interactions to be those from the cDNA Table that pass a
level of `certainty' as specified to the curator and/or
expert system. To assist in the decision-making process, all
relevant data especially that of the translated frame of the
cDNA and corresponding protein domain is related from other

tables and presented in a usable form to the curator and/or
expert system. This presentation allows for easy recognition
and exclusion or correction of basic errors in the data such
as poor quality sequencing, or incorrectly cloned cDNA
fragments. These may include contaminating fragments which
can be identified as originating from an organism which is
different to that of the cDNA library.

A given cDNA is entered into the PathCode_Table only once for
each interaction in which it is found, together with a record
for the corresponding interacting cDNA (or cDNAs for multimer
complexes). However, where a cDNA has different interactions,
for example with different proteins or where different
protein domains of the cDNA interacts with different
proteins, then in each case a different record for the cDNA
is created. These different records are linked by a common
and unique `Interaction ID'. A given interaction is
represented thus only once in the PathCode_Table, and is
related to previous tables in the data-base by the host-cell
clone that represents the interaction and the ID of each cDNA
in the interaction. Said host-cell that represents the
interaction is selected by consideration and curation of all
host-cells and the interacting fragments representing said
interaction held in the cDNA_Table.

A set of criteria can be implemented to assist in said
curation and selection, and to derive a measure of confidence
for the interaction. As way of example, such criteria may
have decreasing information value and include: First, if a
given interaction is observed in both directions of the
experiment ie proteinA-AD interacting with proteinB-BD, and
proteinB-BD interacting with proteinA-DB. Second, if
different examples of the same interaction are observed.
Where different examples of the same interaction are defined
as protein fragments of substantially different length and
position (for example greater than 10% different) but from
the same underlying protein domain and are also found to

interact. Third, if the same examples of the same interaction
are observed, for example by multiple cloning of the same
fragments where the same fragments are of substantially the
same length and position from the same underlying protein
domain. Fourth, that the protein domains that interact may
have biological relevance. That is, similar domains or genes
are known to interact from public literature, or it is known
that both genes are expressed or likely to be expressed in
the same cellular location. This criterion can also be used
as an internal quality control of the library cloning,
interaction experiment and subsequent identification of
interacting members since every interaction experiment should
identify a certain set of published `house-keeping
interactions', and the identification of such interactions
can be used as quality measure for the overall interaction
experiment.

One criterion of particular importance, is the optional
validation of a given interaction by secondary experiments.
For example, cDNA fragments representing the interacting
proteins may be subcloned, and additional interaction
experiments be conducted. Said additional interaction
experiments may include testing each protein for interaction
against a set of unrelated proteins to investigate the
specificity of said interaction. Said testing may be
conducted using the same interaction method that identified
the interaction, for example the yeast two-hybrid, but
preferable it is an independent method. Favoured, is where a
given interaction is biochemically validated using methods
including tissue co-northern, cellular co-localisation or co-
precipitation studies.

All these criteria are considered by the curator and/or
expert system to assist in the decision on which cDNA
fragments and their interactions are entered into the
PathCode_Table. Other interactions known or published in
scientific literature may also be entered into this data-base

during the curation procedure, and hence a field in the table
represents the source of this interaction being internal or
an external reference. The PathCode table has relational
links to secondary or external data-bases holding data on
nucleotide and protein sequences, and biochemical,
structural, biological or bibliographical information. These
data, representing the complete relationships between all
tables and data-bases can be queried by using simple user
interfaces, designed for example using Java, or by more
complicated commands such as those provided by SQL. Possible
queries include those to locate from these data interactions,
pathways or networks for a given nucleotide or amino acid
sequence or motif, or for a given 3-dimensional structure or
motif. Secondly, for highly established networks, these data
may be queried to identify a given pathway between two given
points. It may be that some queries are more efficiently
conducted using a substantially different design of the
PathCode_Table ⅜ for example by representing a given
interaction as the underlying record rather than a given
member of an interaction. A person skilled in the art would
be able to transfer data from one table design to another
using standard data-parsing systems to enable said more
efficient conduction of queries.

The result of these queries is displayed using graphical
methods to enable the investigator to interpret these data
most efficiently. Said graphical methods to include elements
activated by mouse clicks such as hotlinks to seamlessly link
these data with other data sources, or to query and display
further levels of interactions. Computer-based methods of
generating visual representations of specific interactions,
partial or complete protein-protein interaction networks can
be employed to automatically calculate and display the
required interactions most efficiently. Both finding the
network paths and calculating the optimal display of the
found paths can be based on algorithms well known in the art
of mathematical graph theory. For example, algorithms similar

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to those which have been employed to display other biological
relationships such as genetic pedigrees and phylogenetic
relationships.

An established computer data-base of protein interactions has
many useful applications. For example, it may be used to
predict the existence of new biological interactions or
pathways, or to determine links between biological networks.
Furthermore with this method, the function and localisation
of previously unknown proteins can be predicted by
determining their interaction partners. It also can be used
to predict the response of a cell to changes in the
expression of particular members of the networks without
making a molecular, cellular or animal experiment. Finally,
these data can be used to identify proteins or interactions
between proteins within a medically relevant pathway, which
are suitable for therapeutic intervention, diagnosis or the
treatment of a disease.

Example 8:   Preselection against false positive clones and
the automated creation of a regular grid-pattern of yeast
cells expressing a fusion protein

8.1 Genetic pre-selection of false positive clones

Three mating type-a yeast strains were constructed by co-
transformation using the method of Schiestel & Gietz (1989)
into L40ccu, of the plasmid pLUA containing the URA3 readout
system, and either the pBTM117c, pBTM117c-SIM1 or pBTM117c-
HIP1 plasmids respectively. Transformants that contained both
the pLUA plasmid and one of the DNA binding domain plasmid
were selected on SD-trp-ade medium. Three mating type-α yeast
strains were similarly constructed by cotransformation into
L40ccuα of pLUA, and either the pGAD427, pGAD427-ARNT or
pGAD427-LexA plasmids respectively. Transformants that
contained both the pLUA and one of the activation domain

plasmids were selected on SD-leu-ade medium. The yeast
strains thus obtained are listed in Table 3.

The yeast strains x1a, x2a and x3a were replica plated onto
the selective media SD-trp-ade, SD-trp-ade containing 0.2% 5-
FOA and SD-trp-ade-ura, while the yeast strains y1α, y2α and
y3α were replica plated onto the selective media SD-leu-ade,
SD-leu-ade containing 0.2% 5-FOA and SD-leu-ade-ura. Table 4
shows that the two yeast strains x3a and y3α which expressed
the fusion proteins LexA-HIP1 and GAL4ad-LexA respectively
were unable to grow on their respective media containing 5-
FOA yet were able to grow on their respective media lacking
uracil. In contrast, all other yeast strains that contained
plasmids that expressed fusion proteins that were alone
unable to activate the readout system could grow on their
respective media containing 5-FOA, but could not grow on
selective media lacking uracil. This indicates that it is
possible to eliminate yeast clones that express single fusion
proteins which auto-activate the readout system, by selection
on media containing 5-FOA. Thus, the URA3 readout system
successfully eliminated clones containing auto-activating
fusion proteins prior to interaction mating.

8.2 Creation of a regular grid pattern of genetically pre-
selected yeast cells expressing a fusion protein

Two defined libraries of clones that express fusion proteins
were created. First, the yeast strain L40ccu was transformed
with the plasmid pLUA and a resulting stable transformant
colony cultured in minimal medium lacking adenine. Cells from
this culture were rendered competent and transformed with 3
μg pooled mixture of all six pBTM117c constructs shown in
Table 2. Second, the yeast strain L40ccuα was transformed
with the plasmid pLUA and a resulting stable transformant
colony cultured in minimal medium lacking adenine. Cells from
this culture were rendered competent and transformed with 3
μg pooled mixture of all six pGAD427 constructs shown in

Table 2. In all cases, competent cells were prepared and transformations conducted using the method of Schiestel & Gietz (1989).

The two transformation mixes were incubated at 30°C for 2 hours in 10 ml of YPD liquid medium before plating onto large 24 x 24 cm agar trays (Genetix, UK). The *Mata* cells containing the pBTM117c fusion library were plated onto minimal medium lacking tryptophan and adenine but containing 0.2% 5-FOA (SD-trp-ade+FOA), while the *Matα* cells containing the pGAD427 fusion library were plated onto minimal medium lacking leucine and adenine but containing 0.2% 5-FOA (SD-leu-ade+FOA). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 30°C for 4 to 7 days resulting in approximately 1500 colonies per tray.

Mata clones containing the plasmid pBTM117c-HIP1 and *Matα* strains containing the plasmid pGAD427-LexA expressed the fusion proteins LexA-HIP1 and GAL4ad-Lexa respectively. These fusion proteins were shown to activate the URA3 readout system without any interacting fusion protein. Therefore, cells carrying these plasmids should be unable to grow on selective media containing 5-FOA. Hence, only those yeast clones expressing a single fusion protein unable to activate the URA3 reporter gene will form colonies on be picked by the modified robotic system.

Using the modified laboratory picking robot, individual yeast colonies were automatically picked from the agar-trays into individual wells of a sterile 384-well microtiter plates, as described in section 1.3.1 except that the *Mata* yeast strains were picked into microtiter plates containing the growth medium SD-trp-ade and 7% (v/v) glycerol, while the *Matα* yeast strains were picked into microtiter plates containing the growth medium SD-leu-ade and 7% (v/v) glycerol . The resulting microtiter plates were incubated at 30°C for 4 days

with a cell-dispersal step after 36 hours section 3.1. After
incubation, each plate was replicated to create two
additional copies into labelled 384-well microtiter plates
and pre-filled with the liquid growth medium containing 7%
glycerol as was appropriate for the yeast strain. The
replicated plates were incubated at 30 °C for 4 days with a
cell dispersion step conducted after 36 hours as above,
subsequently frozen and stored at -70 °C together with the
original picked microtiter plates of the libraries of cells
expressing fusion proteins.

It will be clear that higher density regular grid-patterns of
such an interaction library can be easily generated by a
person skilled in the art from these microtiter plates of
diploid yeast cells by following the methods disclosed in
sections 3.2, 3.3 and 3.4 of this invention.

8.3   Visual differentiation against false positives for an
improved yeast two-hybrid system

Six yeast strains were generated by transforming each of the
pBTM117c plasmid constructs described in Table 2 into L40ccu
by the method of Schiestel & Gietz (1989). Each strain was
plated on selective growth medium lacking tryptophan,
buffered to pH 7.0 with potassium phosphate and containing 2
ug/ml of the ß-galactosidase substrate X-Gal (SD-trp/XGAL).
Six further strains were similarly constructed by
transforming each of the pGAD427 plasmid constructs described
in Table 2 into L40ccuα. These strains were plated on
selective growth medium lacking leucine, buffered to pH 7.0
with potassium phosphate and containing 2 ug/ml of X-Gal (SD-
leu/XGAL). After incubation at 30 °C for 7 days, the strains
were inspected for growth and blue colour. Table 5 shows that
although all yeast strains were able to grow on the selective
media, only the L40ccu strain expressing the fusion protein
LexA-HIP1 and the L40ccuα strain expressing the fusion
protein GAL4ad-LexA turned blue. In contrast, all other yeast

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strains that contained plasmids that expressed fusion
proteins unable to activate the readout system alone could
grow on the selective media, but did not turn blue. It was
found that for the fusion proteins described here, the blue-
colour generated by auto-activation of the ß-galactosidase
readout system developed faster than any pink-colour of other
clones due to the *ade2* mutation. However, the blue colour may
develop slower than the pink colour for some fusion proteins
that may affect the reliability of visual differentiation
using automated systems with grey-scale vision systems.
Therefore, a person skilled in the art will be able to
incorporate colour recognition systems, colour filters or
construct a yeast strain that does not develop the pink
colour. For example, using a strain carrying the wild-type
ADE2 gene, or the complementary mutation *ade3*.

8.4 Using automation to visually discriminate false-positive
yeast clones and the creation of a regular grid pattern of
cells

Two defined fusion protein libraries were generated. Six
pBTM117c constructs shown in Table 2 were pooled and 3 μg of
the mixture was co-transformed into the yeast strain L40ccu.
The resulting transformants were selected by plating the
mixture onto five large 24 x 24 cm agar-tray (Genetix, UK)
containing minimal medium lacking tryptophan, buffered to pH
7.0 with potassium phosphate and containing 2 ug/ml of X-Gal
(SD-trp/XGAL). Second, the six pGAD427 constructs shown in
Table 5 were pooled and 3 μg of the mixture was co-
transformed into the yeast strain L40ccuα. The resulting
transformants were selected by plating the mixture onto five
large 24 x 24 cm agar-tray (Genetix, UK) containing minimal
medium lacking leucine, buffered to pH 7.0 with potassium
phosphate and containing 2 ug/ml of X-Gal (SD-leu/XGAL).
These agar-trays were poured using an agar-autoclave and pump
(Integra, Switzerland) to minimise tray-to-tray variation in
agar colour and depth. The agar-trays were incubated for 7

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days to allow the yeast clones to grow and the blue colour of
clones able to activate the ß-galactosidase reporter gene to
develop. In all cases, competent cells were prepared and
transformations conducted using the method of Schiestel &
Gietz (1989).

Using the modified laboratory picking robot, individual yeast
colonies were automatically picked from the agar-trays into
individual wells of a sterile 384-well microtiter plates, as
described in section 3.1 except that the Mata yeast strains
were picked into microtiter plates containing the growth
medium SD-trp and 7% (v/v) glycerol, while the Matα yeast
strains were picked into microtiter plates containing the
growth medium SD-leu and 7% (v/v) glycerol .

Automated visual differentiation was made by using the blue-
white sorting parameters described in section 3.1. The robot
was programmed to pick only white colonies into microtiter
plates and ignore all colonies that had turned blue on
activation of the ß-galactosidase reporter gene. Figure 20
displays automated visual discrimination of false positive
clones using the modified picking system described above. The
resulting microtiter plates were incubated at 30°C for 4 days
with a cell-dispersal step after 36 hours section 3.1. After
incubation, each plate was replicated to create two
additional copies into labelled 384-well microtiter plates
and pre-filled with the liquid growth medium containing 7%
glycerol as was appropriate for the yeast strain. The
replicated plates were incubated at 30 °C for 4 days with a
cell dispersion step conducted after 36 hours as above,
subsequently frozen and stored at -70 °C together with the
original picked microtiter plates of the libraries of cells
expressing fusion proteins.

It will be clear that higher density regular grid-patterns of
such an interaction library can be easily generated by a
person skilled in the art from these microtiter plates of

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diploid yeast cells by following the methods disclosed in
sections 3.2, 3.3 and 3.4 of this invention.

Only those colonies that expressed the fusion protein LexA-
HIP1 or the GAL4ad-LexA should be able to activate the LacZ
gene and hence turn blue when grown on the selective medium.
Therefore, blue colonies from the Mata library would be
expected to carry the pBTM117c-HIP1 construct while white
colonies would carry other pBTM117c plasmid constructs.
Likewise, blue colonies from the Matα library would be
expected to carry the pGAD427-LexA construct while white
colonies would carry other pGAD427 plasmid constructs. To
prove this hypothesis, 10 white and 10 blue colonies were
randomly selected from a picked agar-tray of the Mata
library, and twenty colonies from a 384-well microtiter plate
that had been automatically picked from this plate. All 40
colonies were hand inoculated into individual 1ml liquid
cultures of SD-trp medium and the cultures grown for 3 days
at 30°C. The inset carried by each clone was checked by
direct PCR amplification of the pBTM117c insert from the
yeast culture and DNA sequencing by standard protocols. All
ten yeast colonies that had activated the readout system and
turned blue carried the 1.2 Kb HIP1 fragment, while the white
colonies carried the 1.6 Kb HD1.6, the 1.1 Kb SIM insert or
gave no amplification reaction from the non-recombinant
vector. Of the twenty clones selected from the 384-well
microtiter plate which had been automatically visually
differentiated, none carried the 1.2 Kb HIP1 fragment. A
similar experiment of clones manually selected and
automatically picked from the Matα library confirmed that
blue colonies contained the LexA insert from the pGAD427-LexA
construct, and no automatically picked colonies carried this
insert. The pBTM117c-HIP1 plasmid encoded for the LexA-HIP1
fusion protein, and the pGAD427-LexA encoded for the GAL4ad-
lexA fusion protein were known to auto-activate the readout
system without any partner protein. Hence, automatic visual
differentiation has preselected against these false positive

clones and automatically created a regular grid pattern of
yeast clones expressing a single fusion protein unable to
activate the readout system.

Example 9:   Automated interaction mating to combine
genetic elements in yeast cells

9.1 Automated interaction mating on a solid support in
regular pattern

The yeast strains that did not express auto-activating fusion
proteins in section 8.1 were mated using an automated
approach. Each of the yeast strains x1a, x2a, y1α and y2α
was grown in every well of one of four microtiter plates
containing SD-trp-ade medium for the *Mat*a strains and SD-leu-
ade medium for the *Mat*α strains. Each plate was labelled with
a unique barcode and using a spotting robot such as described
by Lehrach *et al.* (1997), the yeast strains x1a and x2a were
transferred in a defined 2 x 2 duplicate pattern with an
inter-spot spacing of 2mm to Hybond-N+ membrane (Amersham)
which had been pre-soaked with YPD medium. The spotting robot
then automatically transferred the yeast strains y1α and y2α
to the same respective spotting positions on each membrane
as, and already containing the x1a and x2a clones. The robot
automatically sterilised the spotting tool, changed the
microtiter plate between each set of clones transferred and
created a data-file in which the spotting pattern produced
and the barcode that had been automatically read from each
microtiter plate was recorded. The spotted membranes were
transferred to YPD plates and incubated for over night at
30°C to allow mating and growth to occur. Each membrane was
assayed for ß-Gal activity using the method of Breeden &
Nasmyth (1985) and was subsequently air dried overnight. A
digital image of each dried filter was captured using a
standard A3 computer scanner and image processed as described
in section 4.1. The processed image was stored on computer
and the identity of clones that expressed ß-Galactosidase was

determined using the image analysis system described in
section 4.1. Figure 21 shows the results of automated
interaction mating between the strains x1a & y1α and x2a &
y2α. Both resulting diploid strains grew on YPD media, yet
only the diploid strain resulting from the interaction mating
of x2a & y2α that contained plasmids encoding the interacting
fusion proteins LexA-SIM1 & GAL4ad-ARNT respectively, showed
a LacZ+ phenotype and turned blue on incubation with X-Gal.
No ß-galactosidase activity was observed for the diploid
strain resulting from the interaction mating between the
strains x1a and y1α that contained plasmids encoding the
proteins LexA and GAL4ad.

9.2   Automated interaction mating based on liquid culture

Two defined libraries of clones which express fusion proteins
were created. First, the yeast strain L40ccu was transformed
with the plasmid pLUA and a resulting stable transformant
colony cultured in minimal medium lacking adenine. Cells from
this culture were rendered competent and transformed with 3
µg pooled mixture of all six pBTM117c constructs shown in
Table 2. Second, the yeast strain L40ccuα was transformed
with the plasmid pLUA and a resulting stable transformant
colony cultured in minimal medium lacking adenine. Cells from
this culture were rendered competent and transformed with 3
µg pooled mixture of all six pGAD427 constructs shown in
Table 2. In all cases, competent cells were prepared and
transformations conducted using the method of Schiestel &
Gietz (1989).

The cells in the two resulting transformation mixes were
allowed to recover by incubation at 30°C in YPD liquid medium
for 2 hours before plating onto large 24 x 24 cm agar trays
(Genetix, UK). The Mata cells containing the pBTM117c fusion
library were plated onto minimal medium lacking tryptophan
and adenine but containing 0.2% 5-FOA (SD-trp-ade+FOA), while
the Matα cells containing the pGAD427 fusion library were

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plated onto minimal medium lacking leucine and adenine but
containing 0.2% 5-FOA (SD-leu-ade+FOA).

The colonies on the agar-trays were grown by incubation at
30°C for 4 to 7 days. To minimise false positives arising
from dormant cells, the colonies on the two agar-trays were
replica-plated onto new agar-trays containing the same
respective selective media as a given original tray using
standard velvet replication. This replication procedure only
transfered cells from the top of a growing colony and thus
reduced the carry over of dormant cells and hence the number
of false positive clones in the yeast two-hybrid system.
These replica agar-trays were incubated at 30°C for 4 to 7
days in order for the yeast cells to grow.

To conduct the liquid interaction mating, the resulting *Mata*
and *Matα* colonies were separately collected off both replica
trays by washing with 20 ml of liquid minimal medium. These
two mixtures of yeast clones were carefully resuspended,
pelleted and washed with sterile distilled water before
incubation in 100 ml of YPD in order to ensure that the cells
in both mixtures were mating competent. The two populations
of mating competent cells were combined in 500 ml of YPD
liquid media contained within a 10 litre flat bottomed flask
and incubated at 30°C with very gentle shaking (< 60 rpm)
overnight to allow interaction mating to proceed. The
resulting mixture of diploid cells was pelleted by gentle
centrifugation at 3000 rpm for 5 min, washed twice with 50 ml
of sterile distilled water and finally, 10 ml of the
resulting cell suspension was plated onto each of five 24 x
24 cm agar-trays containing 300 ml of minimal medium lacking
leucine, trptophan, adenine, histidine and uracil (SD-leu-
trp-ade-his-ura). The agar trays were poured using an agar-
autoclave and pump (Integra, Switzerland) to minimise tray-
to-tray variation in agar colour and depth. After plating,
the colonies were grown by incubating the trays at 30°C for 4
to 7 days.

After incubation, the resulting diploid yeast cells
expressing interacting fusion proteins were automatically
picked using our modified picking system as described in
section 3.1 except that the picked clones were inoculated
into microtiter plates containing the liquid selective medium
SD-leu-trp-ade/7% glycerol. The interaction library
comprising the diploid yeast cells contained in the
microtiter plates were grown by incubation at 30°C as
described in section 3.1. Two further copies of the
interaction library were made into new microtiter plates
containing SD-leu-trp-ade/7% glycerol growth medium, all
plates were individually labelled with a unique barcode and
stored at §70 °C until required for further analysis as
described in section 3.1.

It will be clear that higher density regular grid-patterns of
such an interaction library can be easily generated by a
person skilled in the art from these microtiter plates of
diploid yeast cells by following the methods disclosed in
sections 3.2, 3.3 and 3.4 of this invention. The creation of
high-density regular grid patterns of diploid yeast cells can
be conducted using the procedures as described in earlier
sections. These arrays can be used to assay reporter gene
activity, or for generation of nucleic acid arrays for
hybridisation. Modifications to selective medium may be
required which a person skilled in the art will recognise.

Example 10:   Application of the improved two-hybrid system
to a prokaryotic two-hybrid system

10.1 Strains, readout systems and vectors

Two *E.coli* strains KS1-OR2HF$^+$ and KS1-OR2HF$^-$ were created
that carry the sacB conterselective marker under the control
of the $placO_R2$-62 promoter, and also the tetracycline
selective gene under the control of a second $placO_R2$-62

promoter. Both strains have the sacB counterselective
reporter gene stabley inserted within the *E.coli* chromosome
by knock-out of the arabinose operon to enable arabinaose
controlled inducible promoters to be utilised. The selective
Tet. reporter gene is stabley inserted in within the
chromosome by knock-out of the lactose operon which also
enables a *lacY* counterselective marker to be utilised. Strain
KS1-OR2HF$^+$ was created by transformation of the fertility
conferring F' plasmid into KS1-OR2HF$^-$. KS1-OR2HF$^-$ was created
by site-specific knock-out and insertion of the sacB reporter
gene construct into the arabinose operon of strain KS1-ORTet
by transformation of the plasmid pKO3-araOrsacB and
subsequent selection for stable insertions using the method
of Link et al. (1997) pKO3-araOrsacB was prepared by blunt-
ended ligation of a 1.4 Kb OrsacB fragment into *Stu* I
digested pKO3-ARA to produce an insert of the OrsacB
fragments flanked by 2.5 Kb bp and 1.0 Kb of the 3' and 5'
ends of the *E.coli* arabinose operon respectively. pKO3-ARA
carries the complete arabinose *E.coli* operon which had been
amplified by PCR from E.coli genomic DNA using tailed
primers, digested with *Sal* I and cloned into the *Sal* I site
of pKO3 by standard procedures. The OrsacB fragment was
created by ligating together PCR fragments of the *plac*O$_R$2-62·
promoter and the sacB gene. The *plac*O$_R$2-62 promoter and sacB
PCR fragments were amplifed using standard procedures and
anchor primers which gave rise to complementary overhangs
between the two consecutive fragments which were subsequently
annealed to generate the chimeric sequence (see, for example,
Current Protocols in Molecular Biology, Eds. Ausubel et al.
John Wiley & Sons: 1992) from the plasmids KJ306-31 and pKO3.
The lac promotor derivative *plac*O$_R$2-62 carried by the plasmid
KJ306-31 was prepared by cleaving the plasmid KJ306 with *Hinc*
II and inserting a 31bp linker sequence (Dove et al. 1997).
The strain KS1-ORTet was created by site-specific knock-out
and insertion of a tetracycline reporter gene under the
control of the *plac*O$_R$2-62 promoter into the lactose operon of
strain KS1F$^-$ also by genomic knock-out utilising the pKO3

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system. The tetracycline gene was obtained by PCR of the plasmid pACYC184. Modifications to the above knock-out insertion method were made to make an appropriate pKO3 construct to enable the knock-out insertion of the chimeric tetracycline reporter gene into the lactose operon as will be possible by a person skilled in the art. The *E.coli* strain KS1F⁻ was constructed from KS1 (Dove et al.) by removal of the F' plasmid using standard plasmid curing procedures.

Two vectors, pBAD18-αRNAP and pBAD30-cI were constructed to provide further genetic features to enable the method of invention (Figure 22). The vectors are based on the pBAD series of vectors which provide tight inductive control expression of cloned genes using the promoter from the arabinose operon (Guzman et al., 1995 J. Bact. 177: 4141-4130, and can be maintained in the same *E.coli* cell by virtue of compatible origins of replication. The plasmid pBAD18-αRNAP expresses under the control of the arabiose promoter, fusion proteins between the α amino terminal domain (NTD) of the α-subunit of RNA polymerase and DNA fragments cloned into the multiple cloning site. The presence of this plasmid in kanamycin sensitive cells can be selected by plating on growth medium supplemented with kanamycin, or for its absence by the counterselective *rpsL* allele by plating on media supplemented with streptomycin (Murphy et al. 1995). The plasmid pBAD30-cI expresses under the control of the arabinose promoter, fusion proteins between the λcI protein and DNA fragments cloned into the multiple cloning site. The presence of this plasmid in amplicillin sensitive cells can be selected by plating on growth medium supplemented with amplicillin, or for its absence by the counterselective *lacY* gene by plating on media supplemented with 2-nitrophenyl-ß-D-thiogalactosidase (tONPG) (Murphy et al. 1995). Additionally, the 288 bp oriT sequence enables unidirectional genetic exchange of the pBAD30-cI plasmid and its derivatives from E.coli cells containing the F' fertility factor to F⁻ strains lacking the fertility factor.

The plasmid pBAD18-αRNAP was constructed by cloning a 0.7 Kb DNA fragment encoding the α amino terminal domain (NTD) (residues 1-248) of the α-subunit of RNA polymerase (α-NTD) into *Eco* RI digested pBAD18-CS. The 0.7 Kb α-NTD fragment was isolated by PCR from the plasmid pHTflα (Tang et al., 1994 Genes Dev 8: 3058-3067). The plasmid pBAD18-CS was obtained by site-specific insertion assisted by PCR cloning of the 400 bp coding region and translational start site of the *rpsL* allele into pBAD18-Kan (Guzman et al 1995) before the transcriptional termination signal of the kanamycin gene to enable polycistronic transcription of the counterselective and selective markers. The *rpsL* allele was obtained by PCR amplification of the plasmid pNO1523 (Murphy et al. 1995).

The plasmid pBAD30-cI was constructed by cloning a 730 bp DNA fragment encoding the λcI protein into *Eco* RI digested pBAD30-TCS. The 730 bp fragment encoding the λcI protein was isolated by PCR from the plasmid pACλcI (Dove et al 1997). The plasmid pBAD30-TCS was obtained by site-specific insertion assisted by PCR cloning of the 1.3 Kb coding region and translational start site of the *lacY* gene into pBAD30-T before the transcriptional termination signal of the ampicillin gene to enable polycistronic transcription of the counterselective and selective markers. The *lacY* gene was obtained by PCR amplification of the plasmid pCM10 (Murphy et al. 1995). The plasmid pBAD30-T was obtained by site specific insertion of a 288 bp oriT sequence obtained by PCR from the F' plasmid between the M13 intergenic region and cat' locus of pBAD30 (Guzman et al 1995).

10.2 Detection and identification of interacting proteins using a large-scale and automated prokaryotic two-hybrid system

Generation of a libraries of *E.coli* cells expressing fusion proteins

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The pSport1 plasmid extraction containing the amplified cDNA
library of *Strongylocentrotus purpuratus* described in section
6.1 was used. Approximately 1 μg of the library inserts were
then isolated from the plasmid DNA by *Hind* III/*Sal* 1
digestion and size selective (1 ⨯ 1.5Kb) agarose gel
purification using standard procedures.

The two plasmids pBAD18-αRNAP and pBAD30-cI were prepared by
digestion with *Hind* III/*Sal* 1. The insert mixture that was
isolated as above was split into two equal fractions and 300
ng was ligated with 50 ng of each of the two prepared
plasmids. Following ligation, the pBAD18-αRNAP reaction was
then transformed into competent KS1-OR2HF⁻ *E.coli* cells, and
the pBAD30-cI was transformed into competent KS1-OR2HF⁺
E.coli cells.

Genetic preselection against false positive clones and the
automated creation of a regular grid-pattern of *E.coli* cells
expressing a fusion protein

The two transformation mixes were plated onto large 24 x 24
cm agar trays (Genetix, UK) containing selective media. The
F⁻ cells containing the pBAD18-αRNAP fusion library were
plated onto LB selective medium supplemented with kanamycin
(50 ug/ml), arabinose (0.2% w/v) and sucrose (5% w/v). The F⁺
cells containing the pBAD30-cI fusion library were plated LB
selective medium supplemented with amplicillin (100 ug/ml),
arabinose (0.2%) and sucrose (5%). The agar trays were poured
using an agar-autoclave and pump (Integra, Switzerland) to
minimise tray-to-tray variation in agar colour and depth.
After plating, the colonies were grown by incubating the
trays at 37°C for 18 to 24 hours. The *E.coli* cells expressed
fusion proteins under the control of the arabinose promoter,
and those cells expressing single fusion proteins able to
auto-activate the sacB reporter gene were unable to grow,
since expression of the sacB gene confers sensitivity to

sucrose supplemented in the growth media at high
concentrations.

Automated picking of *E.coli* clones for DNA analysis using
vision-controlled robotic systems such as described in
Lehrach et al. (1997) is well known in the art. Such systems
should also be appropriate for the analysis of *E.coli* cells
that express interacting or potentially interacting fusion
proteins. Therefore, a laboratory picking robot was used to
automatically pick individual *E.coli* colonies from the
selective agar-trays into individual wells of a sterile 384-
well microtiter plate (Genetix, UK) containing sterile liquid
medium. The cells expressing the pBAD18-αRNAP fusion library
were inoculated into liquid LB selective medium supplemented
with kanamycin (50 ug/ml) and 10% (v/v) glycerol
(LB+Kan/10%Gly), while the cells expressing the pBAD30-cI
fusion library were inoculated into LB selective medium
supplemented with amplicillin (100 ug/ml) and 10% (v/v)
glycerol (LB+Amp/10%Gly). The resulting microtiter plates
were incubated at 37°C for 18 to 24 hours, and after growth
of *E.coli* strains within the microtiter plates, each plate
was labelled with a unique number and barcode. The plates
were also replicated to create two additional copies using a
sterile 384-pin plastic replicator (Genetix, UK) to transfer
a small amount of cell material from each well into pre-
labelled 384-well microtiter plates and pre-filled with the
liquid selective medium containing 10% glycerol as was
appropriate for the *E.coli* strain. The replicated plates were
incubated at 37 °C for 18 to 24 hours, subsequently labelled,
frozen and stored at -70 °C together with the original picked
microtiter plates of the libraries of *E.coli* cells expressing
fusion proteins.

In this manner, we generated a regular grid patterns of
E.coli cells expressing fusion proteins using a robotic and
automated picking system. 384-well microtiter plates have a
well every 4.5 mm in a 16 by 24 well arrangement. Therefore,

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for each 384-well microtiter plate we automatically created a
regular grid pattern at a density greater that 4 clones per
square centimetre. It will be clear that higher density
regular grid-patterns of such an interaction library can be
easily generated by a person skilled in the art from these
microtiter plates of *E.coli* cells by following the methods
disclosed in sections 3.2, 3.3 and 3.4 of this invention. For
example, densities of greater than 19 clones per square
centimetre can be obtained by robotic pipetting of clones
into wells of a 1536-well microtiter plate.

Visual differentiation against false positive clones and the
automated creation of a regular grid-pattern of *E.coli* cells
expressing a fusion protein

To demonstrate that visual differentiation against cells that
express single fusion proteins that auto-activate the readout
system could be applied to a prokaryotic two-hybrid system,
the libraries of fusion proteins described in section 10.2.1
were utilised. The two transformation mixes were plated onto
large 24 x 24 cm agar trays (Genetix, UK) containing
selective media. The F$^-$ cells containing the pBAD18-αRNAP
fusion library were plated onto LB selective medium
supplemented with kanamycin (50 ug/ml), arabinose (0.2%) and
X-Gal (2 ug/ml). The F$^+$ cells containing the pBAD30-cI fusion
library were plated LB selective medium supplemented with
amplicillin (100 ug/ml), arabinose (0.2%) and X-Gal (2
ug/ml). The agar trays were poured using an agar-autoclave
and pump (Integra, Switzerland) to minimise tray-to-tray
variation in agar colour and depth. After plating, the
colonies were grown by incubating the trays at 37°C for 18 to
24 hours and to allow any blue colour of colonies to develop.
The *E.coli* cells expressed fusion protein under the control
of the arabinose promoter, and those cells expressing fusion
proteins able to auto-activate the lacZ reporter gene turned
blue by enzymatic reaction of the X-Gal substrate as is well
known in the art.

Using an automated picking system, white *E.coli* cells
expressing single fusion proteins unable to activate the
readout system were automatically visually differentiated
from false positive *E.coli* cells that had turned blue and
only white *E.coli* cells were arrayed in a regular grid
pattern. A standard laboratory picking robot (Lehrach et al.,
1997) was used except that the improvements relating to
reliable sorting of white from blue yeast colonies as
described in section 3.1 was also used to reliably
discriminate between white and blue *E.coli* colonies. White
E.coli colonies from the two sets of agar trays prepared
above were automatically picked and inoculated into the
appropriate selective media in 384-well microtiter plates as
described in section 10.2. It will be recognised by a person
skilled in the art that higher density regular grid patterns
of these clones may easily be formed.

Automated interaction conjugation to combine genetic elements
in *E.coli* cells

It will be clear to a person skilled in the art that
automated interaction mating on a solid support as described
for yeast cells in section 9.1 is equally appropriate for
E.coli cells of different conjugation types that have been
selected by the methods of genetic preselection or visual
differentiation as disclosed in this invention. In such case,
appropriate modifications to the selective media would be
required. However, a person skilled in the art would be able
to recognise and effect said modifications to the selective
media by following the disclosures herein.

To demonstrate an automated approach to interaction
conjugation based on liquid culture, two libraries of clones
that express fusion proteins were prepared as described in
section 10.1. The F⁻ cells containing the pBAD18-αRNAP fusion
library were plated onto LB selective medium supplemented

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with kanamycin (50 ug/ml), arabinose (0.2%) and sucrose (5%).
The F⁺ cells containing the pBAD30-cI fusion library were
plated LB selective medium supplemented with ampicillin (100
ug/ml), arabinose (0.2%) and sucrose (5%).

To conduct the liquid interaction conjugation, the resulting
F⁻ and F⁺ colonies were separately collected off the agar-
trays by washing with 20 ml of liquid LB medium. These two
mixtures of E.coli clones were carefully resuspended,
pelleted and washed with LB. The two populations of cells
were combined in 500 ml of LB liquid media and incubated at
37°C with gentle shaking for 6 hours to allow interaction
conjugation to proceed. The resulting mixture of E.coli cells
was pelleted by gentle centrifugation at 3000 rpm for 5 min,
washed twice with 50 ml of LB liquid media and finally, 10 ml
of the resulting cell suspension was plated onto each of five
24 x 24 cm agar-trays containing 300 ml of the solid LB
selective medium supplemented with ampicillin (100 ug/ml),
kanamycin (50 ug/ml), arabinose (0.2%) and tetracycline (35
ug/ml) (LA+Amp+Kan+Tet+ara). The agar trays were poured using
an agar-autoclave and pump (Integra, Switzerland) to minimise
tray-to-tray variation in agar colour and depth. After
plating, the colonies were grown by incubating the trays at
37°C for 18 to 24 hours.

After incubation, resulting E.coli cells that expressed
interacting fusion proteins grew on the surface of the
selective agar, and were automatically picked using a
laboratory picking system as described in section 10.2 except
that picked clones were inoculated into microtiter plates
containing the liquid LB medium supplemented with ampicillin
(100 ug/ml), kanamycin (50 ug/ml) and 10% (v/v) glycerol
(LB+Amp+Kan/10%Gly). The interaction library comprising the
E.coli cells contained in the microtiter plates were grown by
incubation at 37°C for 18 to 24 hours. Two further copies of
the interaction library were made into new microtiter plates
containing LB+Amp+Kan/10%Glyc growth medium, all plates were

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individually labelled with a unique barcode and stored at ⊗70
°C until required for further analysis as described above. It
will be recognised by a person skilled in the art that higher
density regular grid patterns of these clones may easily be
formed.

Generation of a regular grid pattern of clones from an
interaction library on planar carriers using automation

A high-throughput spotting robot such as that described by
Lehrach et al. (1997) was used to construct porous planar
carriers with a high-density regular grid-pattern of *E.coli*
clones from the defined interaction library contained within
384-well microtiter plates that is described above. The robot
recorded the position of individual clones in the high-
density grid-pattern by the use of a pre-defined duplicate
spotting pattern and the barcode of the microtiter plate.
Individually numbered membrane sheets sized 222 x 222 mm
(Hybond N+, Amersham UK) were pre-soaked in LB medium, laid
on a sheet of 3MM filter paper (Whatmann, UK) also pre-soaked
in LB medium and placed in the bed of the robot. The
interaction library was automatically arrayed as replica
copies onto the membranes using a 384-pin spotting tool
affixed to the robot. Microtiter plates from the first copy
of the interaction library were replica spotted in a `5x5
duplicate' pattern around a central ink guide-spot onto 10
nylon membranes - corresponding to positions for over
27,000clones spotted at a density of over 100 spots per cm2.
The robot created a data-file in which the spotting pattern
produced and the barcode that had been automatically read
from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of
solid agar media in 24 x 24 cm agar-trays. Six membranes were
transferred to LB+Amp+Kan+Tet agar containing 0.2% arabinose
and two each of the remaining membranes were transferred to
either LB agar supplemented with kanamycin (50 ug/ml),

arabinose (0.2%) and tONPG (1 mM) (LB+kan+ara+tONPG) or LB
agar supplemented with amplicillin (100 ug/ml), arabinose
(0.2%) and streptomycin (at an appropriate concentration for
counterselction) (LB+Amp+ara+Sm). The *E.coli* colonies were
allowed to grow on the surface of the membrane by incubation
at 37 °C for 18 to 24 hours.

Detection of the readout system in a regular grid pattern

Two membranes from each of the selective media was processed
to detect ß-galacosidase activity using the method of Breeden
& Nasmyth (1985) and a digital image was captured and stored
on computer as described in section 4.1. Using the image
analysis and computer systems described section 4.1, positive
E.coli clones were identified by consideration of the
activation state of the ß-galactosidase readout system when
clones had been grown on the various selective media.
Positive clones were identified as those that turned blue
after growth on the selective media LB+Amp+Kan+Tet+ara but
not when grown on either of the counterselective media
LB+Kan+ara+tONPG or LB+Amp+ara+Sm.

Identification of individual members of the interaction

A positive *E.coli* clone (identified as 15F09) that expressed
interacting fusion proteins as determined by the computer
systems as described above, was recovered from a stored
frozen copy of the interaction library. Both members
comprising the interaction were recovered by specific PCR
amplification of the insets carried by the pBAD18-αRNAP and
pBAD30-cI plasmids directly from the *E.coli* culture using
plasmid-specific primers. Both members of the interaction
were sequenced by standard procedures, and the information
entered into a data-base as described in Example 7.

As described in section 4.1, high-density arrays of DNA
representing interaction libraries or members comprising

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interaction libraries can be made by transfer to solid
supports by a variety of means. To demonstrate the
applicability of DNA hybridisation to identify *E.coli* clones
carrying plasmids that encode for interacting fusion
proteins, one membrane that had been taken from the
LB+Amp+Kan+Tet+ara growth medium was processed to affix the
DNA carried by the E.coli cells comprising the interaction
library according to the method of Hoheisel et al (1991). The
insert carried by the pBAD30-cI plasmid of clone 15F09 was
radioactively labelled by the method of Feinberg &
Vogelstein, (1983) and used as a hybridisation probe to the
DNA array, and positive signals identified as described in
section 4.1. A clone (22C11) was identified as hybridising to
the probe and was shown to be a positive clone by query of
the data based described in section 4.1. In this manner,
further steps in a protein-protein interaction pathway can be
identified by hybridisation, consideration of reporter gene
activation of hybridisation-positive clones and recovery of
plasmids encoding members comprising these interactions.
Recovery of the plasmids allows further investigation such as
DNA sequencing to identify the members or repeated
hybridisation to identify further steps in the protein-
protein interaction pathway and hence develop protein-protein
interaction networks as described in section 6.6.

Example 11:   Application of the improved two-hybrid system
to a mammalian two-hybrid system

11.1 Strains, readout systems and vectors

The human embryonic kidney fibroblast-derived cell line HEK
293 (or simply 293 cells) is especially suitable for
mammalian 2H studies due to its high susceptibility for DNA
during transfection (Graham, F.L. and Van der Eb, A.J.
(1973), Virol. 54: 536-539; Graham, F.L., Smiley, J., Russel,
W.C. and Nairn, R. (1977), J. Gen. Virol. 36: 59-72). The
cell line is available from ATCC.

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Plasmids carrying teh mammalian readout systems named
pG5E1bEGFPneo, pG5E1bEGFPhyg or pG5E1bEGFPpur are used. These
plasmids contain the TATA element of the adenoviral E1b gene
and five tandem copies of the GAL4 responsive element UAS_G
(5´ CGGAGTACTGTCC TGCG 3') (Sadowski, I., Ma, J.,
Treizenberg, S. and Ptashne, M. (1988), Nature 335: 559-560)
positioned immediately upstream of the coding sequence for
the enhanced green fluorescent protein (EGFP; Yang, T.T.,
Cheng, L. and Kain, S.R. (1996), Nucl. Acids Res. 24 (22):
4592-4593). These reporter plasmids are generated by
replacing the coding sequence for CAT in G5E1bCAT (Dang,
C.V., Barrett, J., Villa-Garcia, M., Resar, L.M.S., Kato,
G.J. and Fearon, E.R. (1991), Mol. Cell. Biol. 11: 954-962)
by the EGFP coding sequence and introducing either a
neomycin, hygromycin or puromycin resistance marker gene
(neo^r, hyg^r or pur^r) using standard subcloning procedures.

The plasmids pMneo1,2,3 or pMhyg1,2,3, which are derived from
pM1,2,3 (Sadowski, I., Bell, B., Broad, P. and Hollis, M.
(1992), Gene 118: 137-141) by insertion of either neo^r or
hyg^r marker gene using standard subcloning procedures, are
series (1,2,3 correspond to three possible reading frames) of
improved Gal4p-fusion vectors derived from the pSG424
plasmid, which was designed for mammalian expression of
fusion proteins that contain the DNA-binding domain of the
yeast Gal4 protein (Sadowski, I. and Ptashne, M. (1989),
Nucl. Acids Res. 17: 7539). This vector contains a polylinker
preceded by coding sequences for Gal4p amino acids 1-147.
Thus, a hybrid reading frame that encodes a Gal4p-fusion
protein can be generated by inserting cDNA sequences into
the polylinker region of pSG424/pM`s. Transcripts of the
hybrid reading frame are inititated from the SV40 early
promoter and their processing is facilitated by the SV40
polyadenylation signal. Alternatively, the hybrid reading
frames are subcloned into pLXSN or any other similar

retroviral vector to allow packaging cell line-aided
infection of target cells.

The plasmids pVP-Nconeo and pVP-Ncohyg are derived from pVP-
Nco vector (Tsan, J., Wang, Z., Jin, Y., Hwang, L., Bash,
R.O., Baer, R. The Yeast Two-Hybrid System, edn 1. Edited by
Bartel, P.L., Fileds, S. New York: Oxford University Press
(1997): 217-232) by insertion of either a neo^r or hyg^r marker
gene using standard subcloning procedures. pVP-Nco in turn is
an improved version of the pNLVP16 plasmid, which was
constructed for the expression of herpes simplex virus
protein VP16-fusion proteins in mammalian cells (Dang, C.V.,
Barrett, J., Villa-Garcia, M., Resar, L.M.S., Kato, G.J. and
Fearon, E.R. (1991), Mol. Cell. Biol. 11: 954-962). A
polylinker sequence is preceded by an artificial reading
frame including the eleven amino-terminal residues of Gal4p
(MKLLSSIEQAC), a nuclear localization signal from the SV40
large T antigen (PKKKRKVD) and the acidic transactivation
domain (amino acids 411-456) of the VP16 protein.
Alternatively, the hybrid reading frames comprising Gal4 (1-
147) and individual sequences of a cDNA library are subcloned
into pLXSN or any other similar retroviral vector to allow
packaging cell line-aided infection of target cells.

11.2 Detection and Identification of Interacting Proteins

A number of monoclonal 293 cell lines stably containing the
pG5E1bEGFPneo-, pG5E1bEGFPhyg or pG5E1bEGFPpur readout system
are generated by the method of calcium phosphate transfection
(Chen, C. and Okayama, H. (1987), Mol. Cell. Biol. 7:2745-
2752), lipofectamine transfection or any other common
transfection method, followed by selection in G418,
hygromycinB (HygB) or puromycin containing medium,
respectively. It is tested subsequently which particular
clone is most appropriate (number of readout system copies
and site(s) of integration into the host chromosomes may

influence expression levels and inducibility of the reporter
gene) for the method of invention.

The selected 293-G5E1bEGFPneo, 293-G5E1bEGFPhyg or 293-
G5E1bEGFPpur reporter cell line is used as a "modified host
cell strain" to perform the method of invention (detection
and identification of interacting proteins).

Two pools representing all three reading frames of the two
vector series pMneo or Mhyg and pVP-Nconeo or pVP-Ncohyg were
prepared by Not 1/Sal 1 digestion and pooling of 1 μg each of
vectors pMneo / pMhyg 1,2,3 and pVP-Nconeo / pVP-Ncohyg 1,2,3
respectively. 300 ng of a cDNA insert mixture that was
isolated as described in section 6.1 was split into two equal
fractions and was ligated with 50 ng of each prepared vector-
series pool. Following ligation, each reaction was then
separately transformed into electro-competent E.coli cells,
and recombinant clones for each library were selected on five
24 x 24 cm plates ampicillin. Approximately 500 μg of the
pVP-Nconeo / pVP-Ncohyg and 500 μg of the pMneo / pMhyg
libraries were extracted from E.coli transformants by washing
off the plated cells and a subsequent QiaPrep plasmid
extraction of the wash mixture as described above. 16 μg of
each vector was used to transfect a 10cm plate of 293 cells.

11.3 Pre-selection against False Positives by visual
differentiation

The pMneo1,2,3 or pMhyg1,2,3 plasmids containing the cDNA
library fused to the Gal4-DNA binding domain were transfected
into the selected 293 reporter cell line. For infection with
retroviruses, designated packaging cell lines are transfected
with the respective retroviral vectors and virus-containing
supernatant from such cultures is then used to infect the
reporter cell line (according to standard protocols; e.g.
Redemann, N., Holzmann, v.Rüden, T., Wagner, E.F.,
Schlessinger, J. and Ullrich, A. (1992), Mol. Cell. Biol. 12:

491-498). Transfection and infection protocols can be
optimized in a way to introduce on average only one plasmid
per cell by adjusting the plasmid concentration for
transfection or the virus titer during infection. Antibiotics
G418 or HygB are employed to select for successfully
transfected/infected reporter cells.

At this stage it is necessary to eliminate those cells that
display a readout system activation as a consequence of only
expressing a DNA-binding domain-fusion protein (in which case
the fusion protein would be referred to as an "auto-
activator"), instead of requiring an appropriate
(interacting) transactivation domain-fusion protein to be
coexpressed. Thus, the resultant polyclonal pool of stably
transfected/infected reporter cells is then subjected to a
preselection screen using the readout system to visually
differentiate cells that express auto-activating fusion
proteins. In the EGFP-based readout system cells expressing
auto-activators can be identified by screening for expression
of EGFP and consequently for the ability of the respective
cells to emit a green fluorescent light (507 nm) upon
stimulation with the appropriate excitatory wavelength (488
nm) (Yang, T.T., Cheng, L. and Kain, S.R. (1996), Nucl. Acids
Res. 24 (22): 4592-4593). Monitoring readout system
activation is either done by eye using a fluorescence
microscope or by an automated detection device. The cells
that activated the GRP reporter system were visually
differentiated and sorted from otehr cells not actiavting the
reporter system using a flouorescent assisted cell sorting
deivce (FACS). Alternatively, elimination of false positive
cells expressing auto-activators is either done manually or
by removal/killing of cells by means of a suction pump or a
micromanipulator or by a detector-linked automated system
employing micromanipulators or a laser ablation device.

After elimination of cells that express autoactivating fusion
proteins, the remaining polyclonal pool of 293 reporter cells

expressing DNA-binding fusion proteins are then subjected to a second transfection /infection step as described above using pVP-Nconeo or pVP-Ncohyg plasmids or respective retroviral derivatives containing the cDNA library fused to the VP16 transactivator sequence. Selection for successfully transfected/infected cells employing antibiotics G418 or HygB is optional here. If selection is desired it has to be made sure that the resistance marker that forms part of the readout system is different from the marker genes on previously transfected/infected vectors. Addition of the antibiotics selecting for the second transfection/infection-vector may be necessary, if the subsequent screening/final selection procedures take several days to complete, in order to prevent loss/diluting out of the plasmids in the absence of selective pressure. A complete selection also eliminates cells that have not been successfully cotransfected (i.e. have not received a pVP-Nco-plasmid), although such cells would not be a major problem (as long as transfection/infection efficiency is high) because they would not be identified by the interaction screening anyway. It is also noteworthy that the longer the cells are kept in culture until cell lysis (and molecular analyses of the interacting proteins and their corresponding cDNA sequences) the more likely it is to loose cDNAs that encode for more or less toxic fusion proteins.

11.4 Automated Identification of Cells Expressing Interacting Proteins

The resulting polyclonal pool of doubly transfected reporter cells is then subjected to visual screening for interacting proteins as described for the visual preselection. Green fluorescent ("positive") cells, indicative of the expression of two interacting proteins were automatically sorted using a FACS system to arrange cells in a regualr grid patternin wells of a mirotitre plate. Subsequently, single cell PCR and DNA sequencing was conducted to identify members comprising

the interactions. Alternatively, the positive cells can be
seeded onto a culture dish in a regular array/grid pattern.
Cells might also be placed one by one into small wells of a
multiwell dish and provided with an appropriate growth
factor-supplemented medium or conditioned medium to allow the
cells to survive and grow in isolation from other cells.

11.5 Double Preselection and Cell Fusion

The cotransfection protocol described above only includes a
single preselection (instead of a double preselection). It
does not include the possibility of a preselection against
false positive clones arising from pVP-Nco (transactivation
domain-cDNA fusion library) plasmids. Although the number of
false positives from pVP-Nco plasmids is usually much lower
than from pM1,2,3 (DNA binding domain-cDNA fusion library)
plasmids , it may under some circumstances be necessary to
apply a double preselection strategy.

To that end two different polyclonal pools of stable cell
lines expressing either members of the pM- or pVP-Nco-cDNA
fusion library are generated by transfection/infection of the
293 reporter cell line and selected by means of the
respective (different) antibiotics (G418 and HygB) as
described above. Both pools of cell lines are then subjected
separately to preselection and elimination of false positive
clones as detailed above.

In order to combine both fusion vectors and their
corresponding expressed fusion proteins in one cell,
individual cells of both pools of cell lines are fused
together using state-of-the-art cell fusion-protocols
involving PEG-facilitated electrofusion as described in Li,
L.-H. and Hui, S.W. (1994), Biophys. J. 67: 2361-2366; Hui,
S.W., Stoicheva, N. and Zhao, Y.-L. (1996), Biophys. J. 71:
1123-1130, and Stoicheva, N. and Hui, S.W. (1994), Membrane
Biol. 140: 177-182. Fusions between one cell of both pools is

desired. For that purpose one cell of each pool is placed
into each well of a multiwell dish as detailed above. After
cell fusion, the combined cells are then subjected to visual
selection. Cells are left on the same dish for visual or
automated screening or collected and sorted by FACS.

11.6 Double Preselection and Cell Fusion Using an Inducible
Expression System

A disadvantage of the above described double preselection
method is that proteins with toxic or anti-proliferative
effects and their corresponding cDNAs will be lost during the
lengthy selection process necessary to establish polyclonal
pools of stable cell lines for both cDNA-fusion library-
sequences. In order to prevent elimination of cDNA sequences
encoding for toxic/ anti-proliferative proteins one can
combine the double preselection strategy with the following
inducible system.

The host cell strain is a 293 cell line which expresses a
tetracycline (Tet)-controlled transactivator (tTA), which is
a fusion of amino acids 1-207 of the tetracycline repressor
(TetR) and the C-terminal activation domain (130 amino acids)
of herpes simplex virus protein VP16. The cell line is called
293 Tet-Off as tTA is able to activate transcription from a
Tet operator sequence (tetO)-controlled gene only in the
absence of Tet. The reverse situation exists in the 293 Tet-
On cell line, which stably expresses a reverse tTA ((r)tTA)
that requires the presence of Tet to induce transcription
from tetO-regulated genes. Both, 293 Tet-Off and 293 Tet-On
cell lines are G418-resistant (neo^r). These cell lines are
available through Clonetech Inc.. tTA plasmids used to
generate 293 Tet-Off and 293 Tet-On cell lines are described
in Gossen, M. and Bujard, H. (1992), Proc. Natl. Acad. Sci.
USA 89: 5547-5551 and in Gossen M. , Freundlieb, S., Bender,
G., Müller, G., Hillen, W. and Bujard, H. (1995), Science
268: 1766-1769.

293 Tet-On or -Off cell lines are then transfected with a
readout system (described in 11.1.) and the reporter cell
lines 293 Tet-On- or -Off-pG5E1bEGFPhyg/pur are generated
through selection in G418 or HygB.

The sequences for the Gal4-DNA binding domain and for the
SV40 nuclear localisation signal/VP16 transactivation domain
(details and references as given in 11.1) are retrieved from
pM and pVP-Nco plasmids and separately subcloned into the
polylinker of pREV-TRE, a retroviral vector (Clonetech Inc.)
to generate pREV-TRE-Gal4 and pREV-TRE-VP16. pREV-TRE
contains the retroviral extended packaging signal, Ψ+, which
allows for production of infectious but replication-
incompetent virus in conjunction with a packaging cell line
such as PT67, followed by a hyg^r gene (selectable marker) and
seven copies of tetO fused to the cytomegalovirus (CMV)
minimal promoter immediately 5´of the polylinker. Ψ+ and
polylinker sequences are flanked by 5´and 3´LTRs,
respectively. pREV-TRE is available from Clonetech Inc.. cDNA
libraries are subcloned into the polylinker of pREV-TRE.
The above described reporter cell lines are separately
infected with either pREV-TRE-Gal4- or pREV-TRE-VP16-derived
retroviral particles. A polyclonal pool of new stable cell
lines is selected in both cases using the resistance
selection marker gene hyg^r. Transient expression of fusion
proteins from pREV-TRE plasmids has to be induced by
withdrawal (Tet-Off) or addition (Tet-On) of Tet in order to
allow for double preselection and elimination of false
positives as described above.

11.7 Cell Fusion and Selection for Cells Expressing
Interacting Proteins

The remaining polyclonal pools of cell lines are then
subjected to cell fusion as described above. The HygB
concentration in the culture medium is increased to minimize

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a possible loss of either one component of the pairs of
fusion protein cDNA sequences present in all fused cells. For
the detection of positive clones, i.e. cells expressing a
pair of interacting proteins (as detailed above), expression
of fusion proteins has to be induced by addition or
withdrawal of Tet.

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Table 1

Oligonucleotide adapters for the construction of the novel yeast two-hybrid vectors pBTM118 a, b and c and pGAD428 a, b and c.

Oligonucleotide	Sequence (5'-3')
a sense	TCGAGTCGACGCGGCCGCTAA
A antisense	GGCCTTAGCGGCCGCGTCGAC
b sense	TCGAGGTCGACGCGGCCGCAGTAA
B antisense	GGCCTTACTGCGGCCGCGTCGACC
c sense	TCGAGAGTCGACGCGGCCGCTTAA
c antisense	GGCCTTAAGCGGCCGCGTCGACTC

Table 2

Two-hybrid vectors used for the expression of fusion proteins.

Plasmid	Fusion-protein	Insert (kb)	Counter-selection	Selection in yeast	Fusion protein Reference
PBTM117c	LexA	-	CAN1	TRP1	N/A
pBTM117c-HD1.6	LexA-HD1.6	1.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-HD3.6	LexA-HD3.6	3.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-SIM1	LexA-SIM1	1.1	CAN1	TRP1	Probst et al., 1997
pBTM117c-MJD	LexA-MJD	1.1	CAN1	TRP1	this work
pBTM117c-HIP1	LexA-HIP1	1.2	CAN1	TRP1	this work
PGAD427	GAL4ad	-	CYH2	LEU2	N/A
pGAD427-ARNT	GAL4ad-ARNT	1.4	CYH2	LEU2	Probst et al., 1997
pGAD427-HIP1	GAL4ad-HIP1	1.2	CYH2	LEU2	Wanker et al., 1997
pGAD427-HIPCT	GAL4ad-HIPCT	0.5	CYH2	LEU2	Wanker et al., 1997
pGAD427-14-3-3	GAL4ad-14-3-3	1.0	CYH2	LEU2	this work
pGAD427-LexA	Gal4ad-LexA	1.2	CYH2	LEU2	this work

Table 3

Yeast strains used for the 5-FOA counterselection and the automated interaction mating

Strain	Plasmids	Selected on
x1a	pBTM117c / pLUA	SD-trp-ade
x2a	pBTM117c-SIM1 / pLUA	SD-trp-ade
x3a	pBTM117c-HIP1 / pLUA	SD-trp-ade
y1α	pGAD427 / pLUA	SD-leu-ade
y2α	pGAD427-ARNT / pLUA	SD-leu-ade
y3α	pGAD427-LexA / pLUA	SD-leu-ade

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Table 4

Identification of fusion proteins that activate the URA3
readout system.

a.

Strain	Plasmids	SD-trp -ade	SD-trp -ade+5- FOA	SD-trp -ade- ura
x1a	pBTM117c / pLUA	+	+	–
x2a	pBTM117c-SIM1 / pLUA	+	+	–
x3a	pBTM117c-HIP1 / pLUA	+	–	+

SD-trp-ade: Selective medium lacking tryptophan and adenine.
SD-trp-ade+5-FOA: Selective medium containing 0.2 % 5-FOA.
SD-trp-ade-ura: Selective medium lacking tryptophan, adenine
and uracil.

b.

Strain	Plasmids	SD-leu -ade	SD-leu -ade+5-FOA	SD-leu -ade-ura
y1α	pGAD427 / pLUA	+	+	–
y2α	pGAD427 -ARNT/pLUA	+	+	–
y3α	pGAD427 -LexA/pLUA	+	–	+

SD-leu-ade: Selective medium lacking leucin and adenine.
SD-leu-ade+5-FOA: Selective medium containing 0.2 % 5-FOA.
SD-leu-ade-ura: Selective medium lacking leucin, adenine and
uracil.

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Table 5

Identification of fusion proteins that activate the LacZ
readout system.

A. L40ccu yeast cells transformed with pBTM117c plasmid
constructs expressing a fusion protein comprising the LexA
DNA binding domain are plated on minimal medium lacking
trptophan, buffered to pH 7.0 with potassium phosphate and
containing 2 ug/ml of X-Gal (SD-trp/XGAL): Results for the
state of the readout system for various auto-activating and
non-auto-activating fusion proteins

Plasmid Construct	Fusion protein	Growth on SD-trp/XGAL	Blue colouration
pBTM117c	LexA	+	-
pBTM117c-HD1.6	LexA-HD1.6	+	-
pBTM117c-HD3.6	LexA-HD3.6	+	-
pBTM117c-SIM1	LexA-SIM1	+	-
pBTM117c-MJD	LexA-MJD	+	-
pBTM117c-HIP1	LexA-HIP1	+	+

B. L40ccuα yeast cells transformed with pGAD427 plasmid
constructs expressing a fusion protein comprising the GAL4ad
activation domain are plated on minimal medium lacking
leucine, buffered to pH 7.0 with potassium phosphate and
containing 2 ug/ml of X-Gal (SD-leu/XGAL): Results for the
state of the readout system for various auto-activating and
non-auto-activating fusion proteins.

Plasmid Construct	Fusion protein	Growth on SD-leu/XGAL	Blue colouration
pGAD427	GAL4ad	+	-
PGAD427-ARNT	GAL4ad-ARNT	+	-
PGAD427-HIP1	GAL4ad-HIP1	+	-
PGAD427-HIPCT	GAL4ad-HIPCT	+	-
PGAD427-14-3-3	GAL4ad-14-3-3	+	-
PGAD427-LexA	Gal4ad-LexA	+	+

CLAIMS

1.   A method for the identification of at least one member
     of a pair or complex of interacting molecules from a
     pool of potentially interacting molecules, comprising:

     (A)   providing at least one set of host cells, each set
     containing at least one genetic element comprising a
     selectable marker, said selectable marker being
     different between different sets of host cells, said
     genetic elements each comprising genetic information
     specifying one of said potentially interacting
     molecules, said host cells further carrying a readout
     system that is activated upon the presence of auto-
     activating molecules;

     (B)   selecting against host cells expressing a molecule
     able to auto-activate the readout system by transferring
     at least one set of host cells or progeny of at least
     one set of host cells to at least one selective medium
     which allows growth of said host cells in the presence
     of said selectable marker different for each set of host
     cells and which precludes growth of said host cells upon
     auto-activation of said readout system;

     (C)   combining in host cells at least two genetic
     elements, wherein at least one set of host cells grows
     on said selective medium specified in (B);

     (D)   allowing at least one interaction, if any, to
     occur;
     (E)   selecting for said interaction by transferring said
     host cells or progeny of said host cells to a selective

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medium that allows identification of said host cells
upon activation of the readout system;

(F)   identifying host cells that contain interacting
molecules that activate said readout system on said
selective medium;

(G)   identifying at least one member of said pair or
complex of interacting molecules;

wherein said host cells are not yeast cells.

2.   A method for the identification of at least one member
of a pair or complex of interacting molecules from a
pool of potentially interacting molecules, comprising:

(A)   providing at least one set of host cells, each set
containing at least one genetic element comprising a
selectable marker, said selectable marker being
different between different sets of host cells, said
genetic elements each comprising genetic information
specifying one of said potentially interacting
molecules, said host cells further carrying a readout
system that is activated upon the presence of auto-
activating molecules;

(B)   selecting against host cells expressing a molecule
able to auto-activate the readout system by transferring
at least one set of host cells or progeny of at least
one set of host cells to at least one selective medium
which allows growth of said host cells in the presence
of said selectable marker different for each set of host
cells and visual differentiation between those cells
whose readout system has been activated from those host
cells whose readout system has not been activated;

(C)   combining in host cells at least two different genetic elements, wherein at least one set of host cells grows on said selective medium specified in (B);

(D)   allowing at least one interaction, if any, to occur;

(E)   selecting for said interaction by transferring said host cells or progeny of said host cells to a selective medium that allows identification of said host cells upon activation of the readout system;

(F)   identifying host cells that contain interacting molecules that activate said readout system on said selective medium;

(G)   identifying at least one member of said pair or complex of interacting molecules

3.   A method for the identification of at least one member of a pair or complex of interacting molecules from a pool of potentially interacting molecules, comprising:

(A)   providing at least one set of host cells, each set containing at least one genetic element comprising a selectable marker, said selectable marker being different between different sets of host cell, said genetic elements each comprising genetic information specifying one of said potentially interacting molecules, said host cells further carrying a readout system that is activated upon the presence of auto-activating molecules;

(B)   selecting against host cells expressing a molecule able to auto-activate the readout system by transferring at least one set of host cells or progeny of at least one set of host cells to at least one selective medium

which allows growth of said host cells in the presence
of said selectable marker different for each set of host
cells and which precludes growth of said host cells upon
auto-activation of said readout system;

(C)   combining in host cells at least two genetic
elements, wherein at least one set of host cells grows
on said selective medium specified in (B);

(D)   allowing at least one interaction, if any, to
occur;

(E)   selecting for said interaction by transferring said
host cells or progeny of said host cells to a selective
medium that allows identification of said host cells
upon activation of the readout system;

(F)   identifying host cells that contain interacting
molecules that activate said readout system on said
selective medium;

(G)   identifying at least one member of said pair or
complex of interacting molecules;

wherein said host cells are yeast cells, and at least
one of the steps (B), (C), (E) or (F) is effected or
assisted by automation using regular grid patterns of
host cells.

4.   The method of claims 1 to 3, wherein said pair or
      complex of interacting molecules is selected from the
      group consisting of RNA-RNA, RNA-DNA, RNA-protein, DNA-
      DNA, DNA-protein, protein-peptide, peptide-peptide or
      protein-protein interactions.

5.   The method of claims 1 to 4, wherein said genetic elements are plasmids, artificial chromosomes, viruses or other extrachromosomal elements.

6.   The method of claims 1 to 5, wherein said interaction leads to the formation of a transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a response moiety driving the activation of said readout system.

7.   The method of claims 1 to 6, wherein said readout system comprises at least one counterselectable gene.

8.   The method of claim 7, wherein said counterselectable gene is one of the genes URA3, LYS2, sacB, CAN1, CYH2, rpsL, or lacY.

9.   The method of claims 7 or 8, wherein the selective medium in step (B) comprises a counterselective compound.

10.   The method of claim 9, wherein said counterselective compound is 5-fluoro orotic acid, canavanine, cycloheximide, sucrose, tONPG or streptomycin.

11.   The method of claims 2 and 7 to 10, wherein said readout system comprises or further comprises at least one detectable protein.

12.   The method of claim 11, wherein said detectable protein is encoded from at least one of the genes lacZ, HIS3, URA3, LYS2, tetA, sacB, gfp, yfp, bfp, cat, luxAB, HPRT bla, kan or a surface marker.

13.   The method of claims 1 to 12, wherein said host cells are bacterial cells, mammalian cells, insect cells or plant cells.

14. The method of claims 1 to 13 further comprising
     transforming, infecting or transfecting at least one set
     of host cells of said sets of host cells with said
     genetic element or genetic elements prior to step (D).

15. The method of claims 1 to 13 further comprising
     transforming, infecting or transfecting each set of host
     cells of said sets of host cells with said genetic
     elements prior to step (D).

16. The method of claims 1 to 13 further comprising
     transforming, infecting or transfecting one set of host
     cells of said sets of host cells with at least one
     genetic element prior to step (A), selecting against
     host cells in said one set of host cells expressing a
     molecule able to auto-activate said readout system as
     specified in step (B), and transforming, infecting or
     transfecting said set of host cells with at least one
     further genetic element in step (D).

17. The method of claims 1 to 15, wherein cell fusion,
     conjugation or interaction mating is used for the
     generation of said host cells with said genetic elements
     prior to step (D), preferably in step (C).

18. The method of claim 17, wherein said cell fusion,
     conjugation or interaction mating is affected or
     assisted by automation.

19. The method of claim 18, wherein said automation is
     effected by an automated picking, spotting, rearraying
     pipetting, micropipetting or cell sorting device.

20. The method of claim 19, wherein said device is a picking
     robot, spotting robot, rearraying robot, pipetting

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system, micropipetting system or fluorescent assisted cell sorting (FACS) system.

21. The method of claims 1 to 20, wherein said selectable marker is an auxotrophic or antibiotic marker.

22. The method of claim 21, wherein said auxotrophic or antibiotic marker is LEU2, TRP1, URA3, ADE2, HIS3, LYS2, kan, bla, Zeocin, neomycin, hygromycin, pyromycin or G418.

23. The method of claims 1 to 22, wherein host cells or progeny of host cells of step (D) are transferred to a storage compartment.

24. The method of claim 23, wherein the transfer to a storage compartment is effected or assisted by automation.

25. The method of claim 23 or 24, wherein the transfer to a storage compartment is effected by an automated arraying, replicating, picking, spotting, pipetting or micropipetting or cell sorting device.

26. The method of claim 25, wherein said device is a picking robot, spotting robot, pipetting system, micropipetting system or fluorescent assisted cell sorting (FACS) system.

27. The method of claims 23 to 26, wherein said storage compartment comprises an anti-freeze agent.

28. The method of claims 23 to 27, wherein said storage compartment is at least one microtitre plate.

29. The method of claim 28, wherein said at least one microtitre plate comprises 96, 384, 846 or 1536 wells.

30.   The method of claims 1 to 29, wherein the transfer of
       host cells or progeny of host cells in step (E) is
       effected or assisted by automation using a regular grid
       pattern.

31.   The method of claim 30, wherein the transfer of host
       cells or progeny of host cells in step (E) is effected
       by an automated replicating, picking, spotting,
       pipetting or micropipetting or cell sorting device.

32.   The method of claim 31, wherein said device is a
       replicating robot, picking robot, spotting robot,
       pipetting system, micropipetting system or fluorescent
       assisted cell sorting (FACS) system.

33.   The method of claims 30 to 32, wherein the transfer of
       host cells or progeny of host cells in step (E) is made
       by multiple transfers carrying additional host cells to
       the same position in said regular grid pattern.

34.   The method of claims 1 to 33, wherein the transfer of
       host cells or progeny of host cells in step (E) is made
       to at least one carrier.

35.   The method of claim 34, wherein said at least one
       carrier is a microtitre plate and the regular grid
       pattern is at densities greater than 1, preferably
       greater than 4, more preferably greater than 10, most
       preferably greater than 18 clones per centimeter square.

36.   The method of claim 34, wherein said at least one
       carrier is a porous support and the regular grid pattern
       is at densities in the range of 1 to 10, preferably 10
       to 50, more preferably 50 to 100, most preferably
       greater than 100 clones per centimeter square.

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37. The method of claim 34, wherein said at least one
     carrier is a non-porous support and the regular grid
     pattern is at densities in the range of 1 to 100,
     preferably 100 to 500, more preferably 500 to 1000, most
     preferably greater than 1000 clones per centimeter
     square.

38. The method of claims 1 to 37, wherein the identification
     of host cells in step (F) from consideration of the
     activation state of said readout system using visual
     means is effected or assisted by automation.

39. The method of claims 1 to 38, wherein the identification
     of host cells in step (F) from consideration of the
     activation state of said readout system is effected or
     assisted by automated digital image capture, storage,
     processing, and/or analysis.

40. The method of claims 1 to 39, wherein the identification
     of said at least one member of said pair or complex of
     interacting molecules in step (G) is effected by nucleic
     acid hybridisation, oligonucleotide hybridisation,
     nucleic acid or protein sequencing, restriction
     digestion, spectrometry or antibody reactions.

41. The method of claims 1 to 40, wherein the identification
     of said at least one member of said pair or complex of
     interacting molecules in step (G) is effected using a
     regular grid pattern of said at least one member or of
     said genetic information encoding said at least one
     member.

42. The method of claim 41, wherein construction of regular
     grid patterns in step (G) is effected or assisted by
     automation.

43. The method of claim 42, wherein the automation is
     effected or assisted by an automated spotting, pipetting
     or micropipetting or cell sorting device.

44. The method of claim 43, wherein automation in step (G)
     is implemented by employing a spotting robot, spotting
     tool, pipetting system or micropipetting system.

45. The method of claims 41 to 44, wherein said
     identification is effected by automated digital image
     capture, storage, processing and/or analysis.

46. The method of claims 1 to 45, wherein nucleic acid
     molecules, prior to said identification in step (G), are
     amplified by PCR or are amplified in a different host
     cell as a part of said genetic elements, preferably in
     bacteria and most preferably in E. coli.

47. The method of claims 1 to 46, further comprising:

     (H) providing at least one of said genetic elements in
     step (A), which additionally comprises or comprise a
     counterselectable marker, wherein said counterselectable
     markers are different for each type of genetic element;

     (I) selecting for interaction by transferring host
     cells or progeny of host cells in step (E) to

     (i) at least one selective medium that precludes growth
     of host cells in the presence of the counterselectable
     marker specified in (H) and allows growth in the
     presence of a selectable marker; and

     (ii) a further selective medium that allows
     identification of host cells upon activation of the
     readout system;

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(J)   identifying host cells in step (F) that contain
interacting molecules that:

(iii)   do not activate said readout system on said at
least one selective medium specified in (i); and

(iv) activate said readout system on said selective
medium specified in (ii).

48.   The method of claim 47, wherein the genetic element that
additionally comprises a counterselectable marker
further specifies an activation domain fusion protein.

49.   The method of claims 1 to 46, further comprising:

(K)   providing at least two of said genetic elements in
step (A), which additionally comprise different
counterselectable markers;

(L)   selecting for interaction by transferring host
cells or progeny of host cells in step (E) to

(v)   at least one selective medium, wherein said
selective medium that precludes growth of host cells in
the presence of the first counterselectable marker of
the counterselectable markers specified in (K) and
allows growth in the presence of a first selectable
marker;

(vi) at least one selective medium, wherein said
selective medium precludes growth of host cells in the
presence of the second counterselectable marker of the
counterselectable markers specified in (K) and allows
growth in the presence of a second selectable marker;

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(vii)   a further selective medium that allows
identification of said host cells upon activation of the
readout system; and

(M)   identifying host cells that contain molecules that:

(viii)   do not activate said readout system on said at
least one selective medium specified in (v); and

(ix) do not activate said readout system on said at
least one selective medium specified in (vi); and

(x)   activate said readout system on said selective
medium specified in (vii).

50.   The method of claim 49, wherein said at least two
genetic elements that additionally comprise a
counterselectable marker further specify a DNA binding
domain fusion protein and an activation domain fusion
protein, respectively.

51.   The method of claims 47 to 50, wherein said
counterselectable marker or counterselectable markers of
step (H) or (K) are selected from the group of URA3,
LYS2, sacB, CAN1, CYH2, rpsL or lacY.

52.   The method of claims 47 to 51, wherein the transfer of
host cells or progeny of host cells in step (I) or (L)
is effected or assisted by automation.

53.   The method of claim 52, wherein the said automation in
step (I) or (L) is effected by an automated replicating,
picking, spotting, pipetting or micropipetting or cell
sorting device.

54.   The method of claim 53, wherein said automation in step
(I) or (L) is implemented by employing a replicating

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robot, picking robot, spotting robot, spotting tool,
automated pipetting or micropipetting system, or
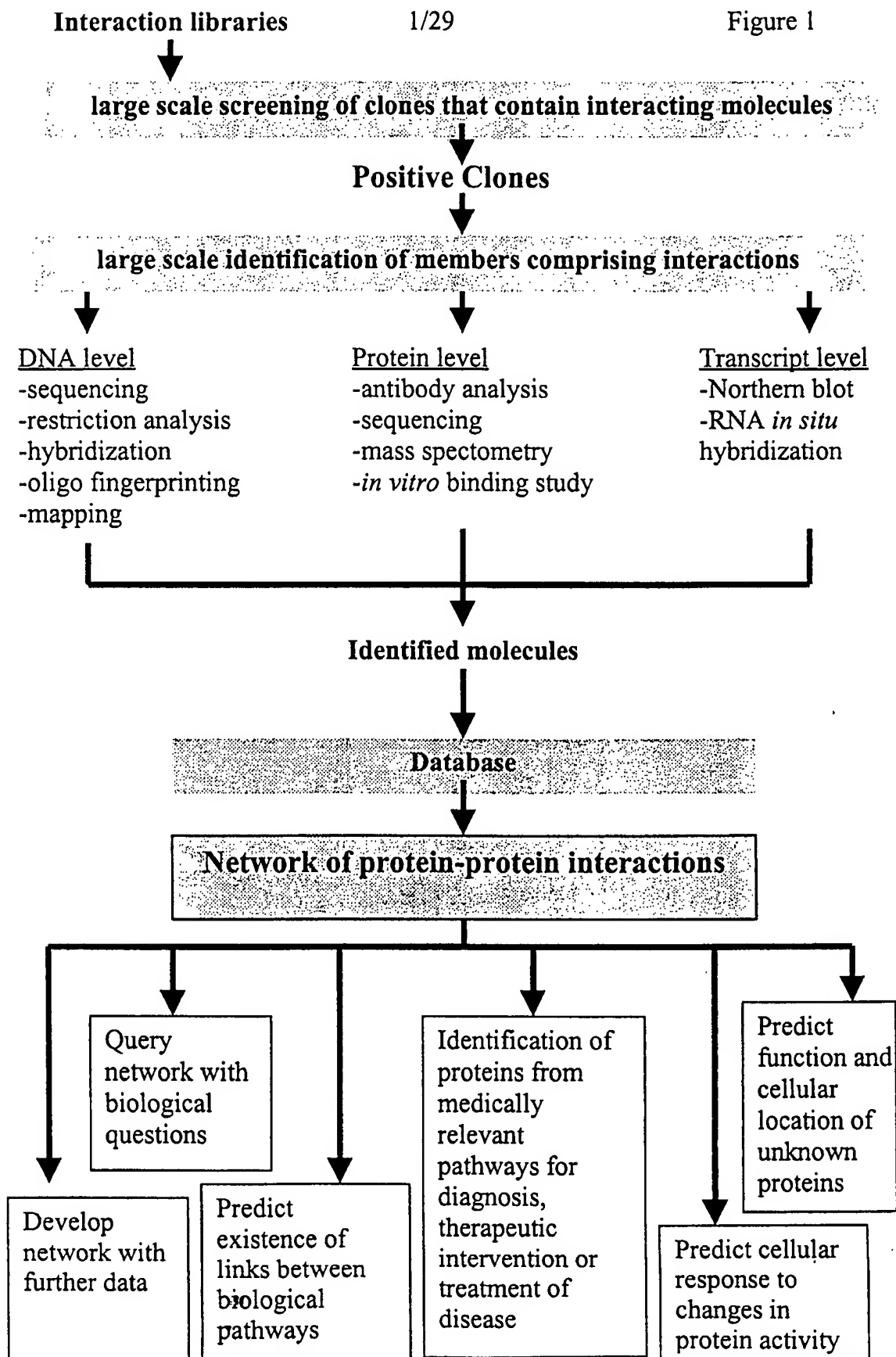fluorescent assisted cell sorting (FACS) system.

55. The method of claim 2, wherein said visual
    differentiation in step (B) is based on a difference
    between host cells in different activation states of the
    readout system which can be detected by visual means.

56. The method of claim 55, wherein said difference between
    host cells in different activation states that can be
    detected by visual means is brought about by activation
    of one of the genes lacZ, gfp, yfp, bfp, CAT, luxAB, or
    of a surface marker.

57. The method of claims 55 or 56, wherein said visual means
    include digital image capture, storage, processing
    and/or analysis.

58. The method of claims 1 to 57, wherein said genetic
    information specifying one of said potentially
    interacting molecules is different for each host cell in
    a set of host cells or a majority of host cells in a set
    of host cells.

59. The method of claim 58, wherein said genetic information
    specifying one of said potentially interacting molecules
    is identical in not more than 10 %, preferably not more
    than 5 %, more preferably not more than 2 %, most
    preferably not more than 1 % of host cells in a set of
    host cells.

60. A method for the production of a pharmaceutical
    composition comprising formulating at least one member
    of the interacting molecules identified by the methods
    of claims 1 to 59 in a pharmaceutically acceptable form.

61.   A method for the production of a pharmaceutical
      composition comprising formulating an inhibitor of the
      interaction of the interacting molecules identified by
      the methods of claims 1 to 59 in a pharmaceutically
      acceptable form.

62.   A method for the production of a pharmaceutical
      composition comprising identifying a further molecule of
      a cascade of interacting molecules of which at least one
      of said interacting molecules identified by the methods
      of claims 1 to 59 is a part of or identifying an
      inhibitor of the function of said further molecule.

63.   Kit comprising:

      (N) Host cells, comprising a readout system which
      allows host cells to be counterselected against auto-
      activation of said readout system; and

      (O) at least one genetic element comprising a
      selectable marker, a counterselectable marker and
      genetic information encoding an activation domain or a
      DNA binding domain, which activation domain and DNA
      binding domain are together able to activate said
      readout system;

           wherein said host cells are not yeast cells.

64.   Kit according to claim 63, wherein said host cells are
      bacterial cells.

65.   Kit comprising:

(P)   Host cells, comprising a readout system which allows
      host cells to be visually differentiated upon activation
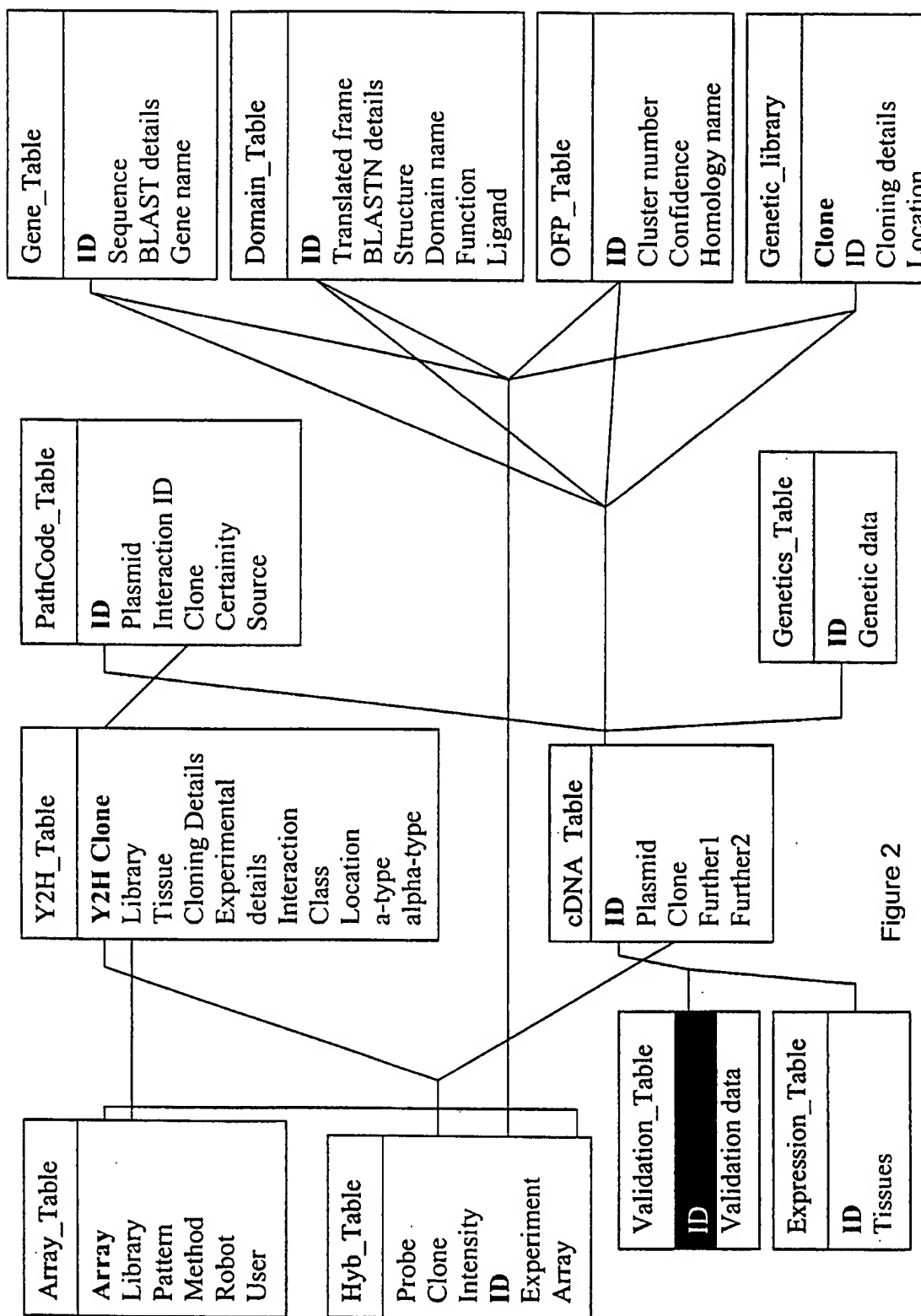   of said readout system; and

(Q)   at least one genetic element comprising a selectable
      marker and genetic information encoding an activation
      domain or a DNA binding domain, which activation domain
      and DNA binding domain are together able to activate
      said readout system;

66.   Kit according to claim 65, wherein said host cells are
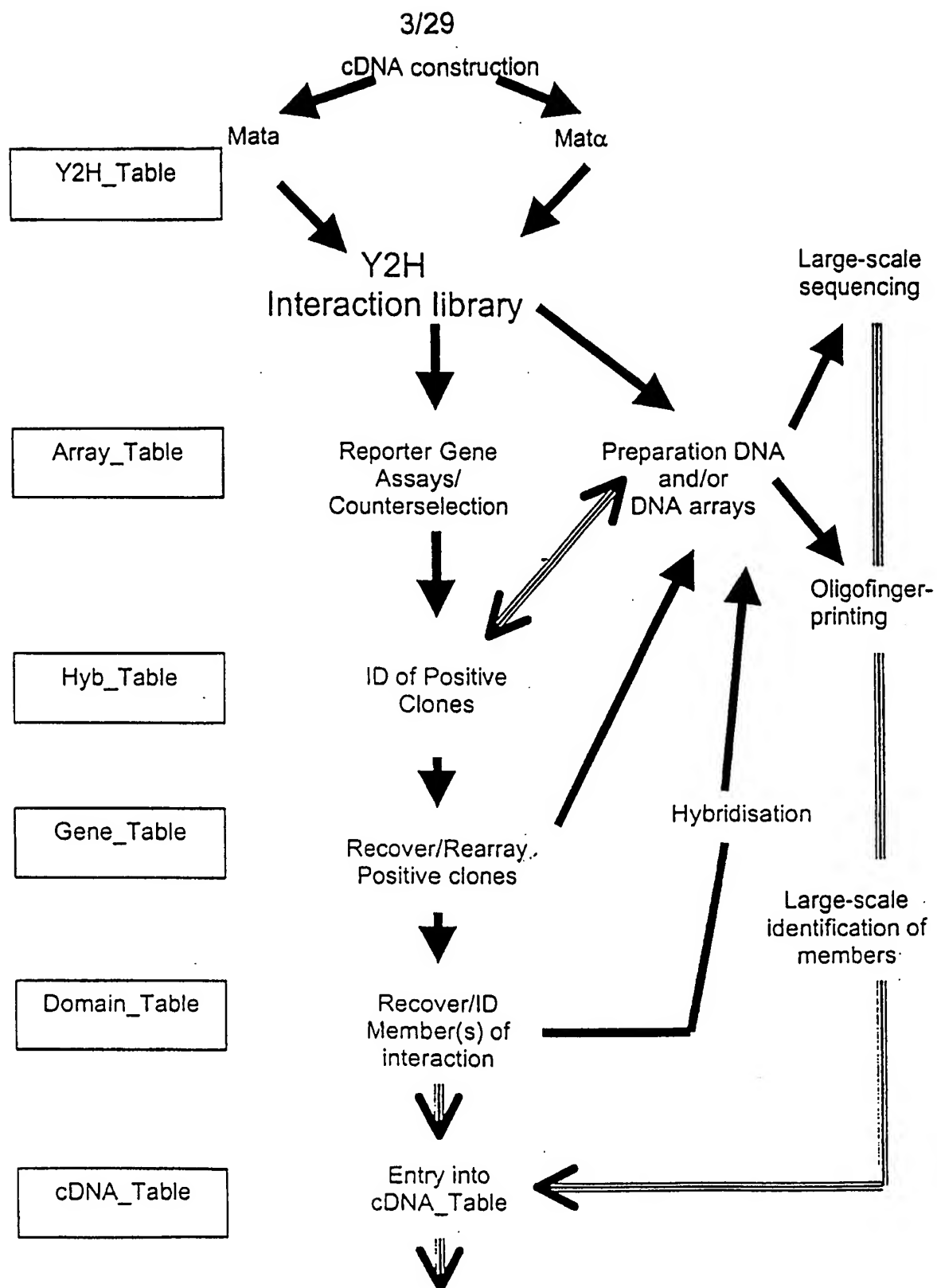   bacterial cells or mammalian cells.

Interaction libraries   1/29   Figure 1

large scale screening of clones that contain interacting molecules

Positive Clones

large scale identification of members comprising interactions

DNA level
-sequencing
-restriction analysis
-hybridization
-oligo fingerprinting
-mapping

Protein level
-antibody analysis
-sequencing
-mass spectometry
-*in vitro* binding study

Transcript level
-Northern blot
-RNA *in situ*
hybridization

Identified molecules

Database

Network of protein-protein interactions

Query network with biological questions

Develop network with further data

Predict existence of links between biological pathways

Identification of proteins from medically relevant pathways for diagnosis, therapeutic intervention or treatment of disease

Predict function and cellular location of unknown proteins

Predict cellular response to changes in protein activity

Figure 2

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(continuation from the previous page)

Published
Interactions

Data
Curation

External Biological
databases

PathCode_Table

PathCode_Table

External Sequence
Databases

Query
Tools/Interfaces

Data flow or informatic
process

Figure 3(II)

Data
Output/Visualisation

Experimental flow or
molecular biological
process

a

Sal I
Not I

ADH1
2u
LexA
T
bla
pBTM118a, b and c
9.50 Kb
TRP1
ori
apha
CAN1

b

Eco RI
Sal I
Not I

ADH1
2u
GAL4ad
T
pGAD428a, b and c
9.50 Kb
LEU2
aphA
CYH2
ori

Figure 4 (I)

b

a) TCG A|GT CGA C|GC GGC CGC| TAA| CCG G

           Sal I   *Not* I   STOP

b) TCG A|GG TCG AC|G CGG CCG C|AG |TAA| CCG G

           Sal I   *Not* I   STOP

c) TCG AGA |GTC GAC| |GCG GCC GC|T |TAA| CCG G

           Sal I   *Not* I   STOP

Figure 4 (II) ↑
Figure 5 ↓

pLUA
9.10 Kb

lexAop
URA3
2u
bla
ADE2
ori

DNA Fragments (eg cDNA library)

DNA Binding
Domain
(eg pBTM118c)

Activation Domain
(eg pGAD428c)

TRP1   CAN1   CYH2   LEU2

Transformation into Mat α-   Mat α-strain

Mating or Cotransformation

His-Selection

SD
-leu-trp-his

Picking the clones using a robotic device

Culturing the cells in 384 microtiter
plates containing selective medium

Replication and storage of
the interaction library

Figure 6(I)

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(continuation from *figure 6 (I)*)

Spotting of yeast cells onto a nylon membrane;
Transfer of these membranes to different selective media

ß-Gal-assay

SD -trp +CHX

SD -leu +CAN

SD -leu-trp-his

Identification of clones that contain interacting molecules
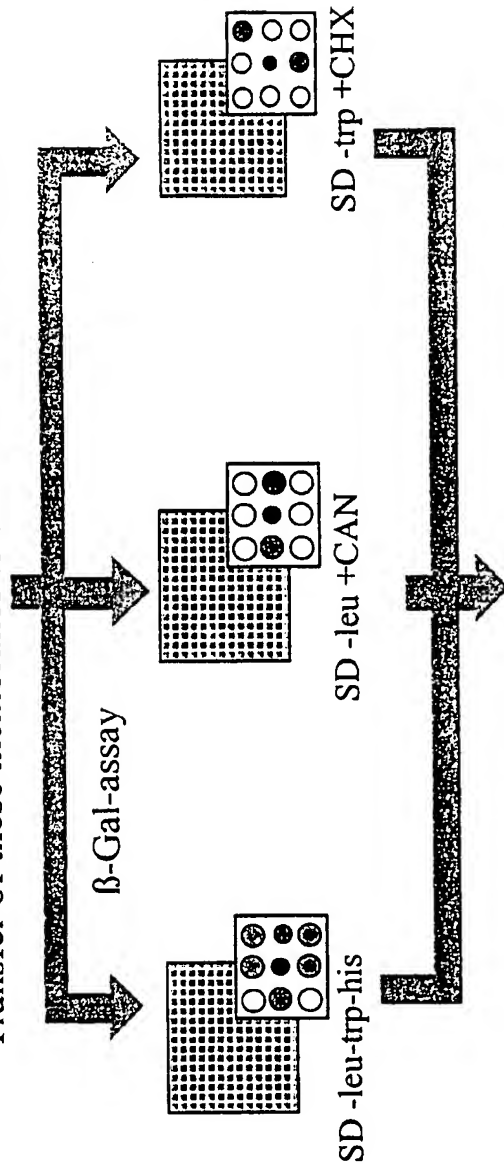
(continuation see figure 6(III))

Figure 6 (II)

Figure 6 (III)

Genetic libraries (e.g. a cDNA library)

DNA Binding Domain

Activation Domain

TRP1   CAN1   CYH2   LEU2

Yeast cells (Mat a)   Yeast cells (Mat α)

Plating onto selective media containing 5-fluoroorotic acid (5-FOA)

a   surviving cells   surviving cells   **b**

Pooling

Picking the clones using a robotic device

Interaction mating

Mata   Matα

384 microtitre
plates

SD
-leu-trp-his-ura

Picking the clones using a robotic
device

Culturing the cells in 384 microtiter
plates containing selective medium

Automated interaction
mating using a spotting
robot or a micropipetting
device

Replicating

Transfer of yeast cells
using a spotting robot to
a nylon membrane

Figure 7(I)

Figure 7 (II)

(continuation from *figure 7 (I)*)
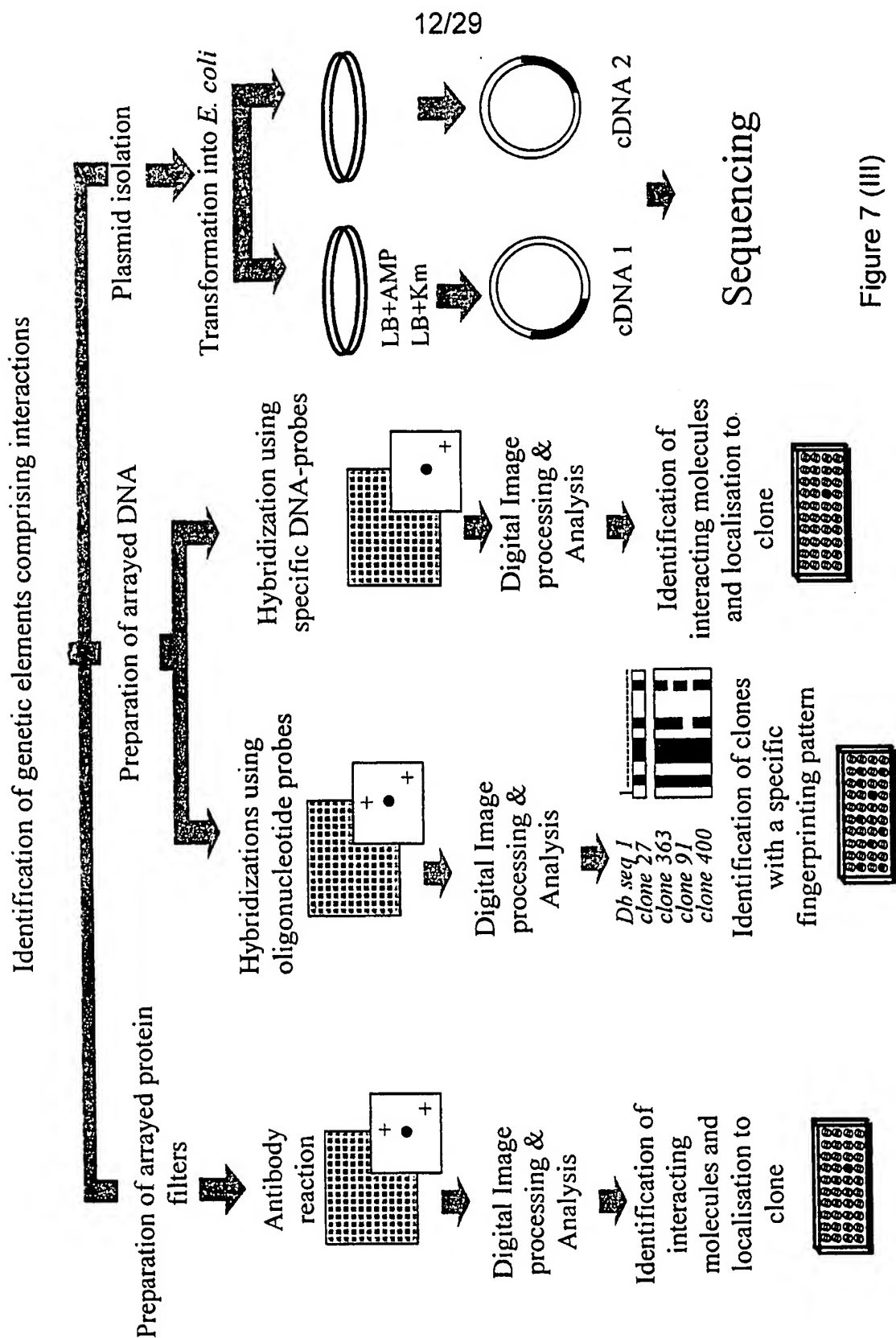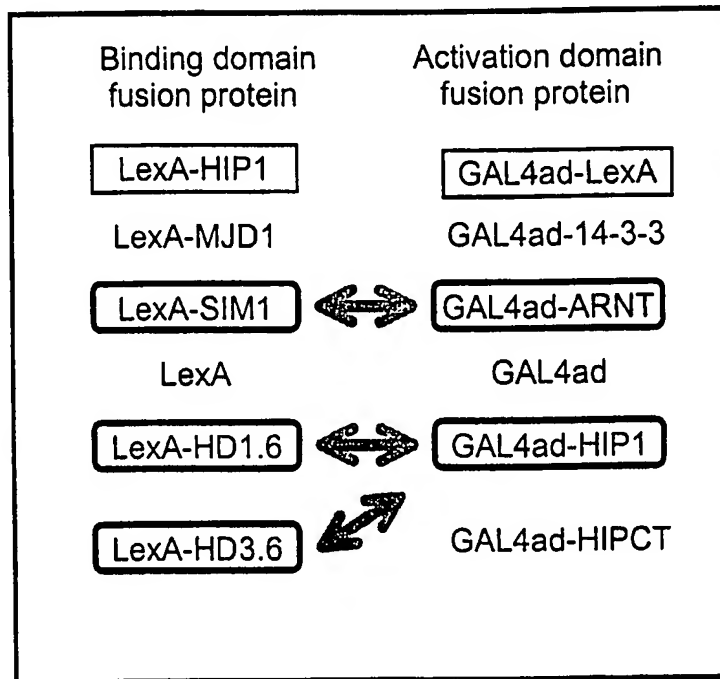
ß-Gal-assay

SD -trp +CHX

SD -leu +CAN

SD -leu-trp-his

Identification of clones that contain interacting molecules

(continuation see figure 7(III))

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Figure 7 (III)

Figure 8

growth   ß-gal assay

SD-leu-trp

pBTM117c-SIM1 + pGAD427-ARNT

pBTM117c + pGAD427

pBTM117c-HIP1 + pGAD427

SD-leu-trp-his

pBTM117c-SIM1 + pGAD427-ARNT

pBTM117c + pGAD427

pBTM117c-HIP1 + pGAD427

SD-leu+CAN

pBTM117c-SIM1 + pGAD427-ARNT

pBTM117c + pGAD427

pBTM117c-HIP1 + pGAD427

SD-trp+CHX

pBTM117c-SIM1 + pGAD427-ARNT

pBTM117c + pGAD427

pBTM117c-HIP1 + pGAD427

Figure 9

Figure 10 (I)

Figure 10 (II)

Figure 11

a.
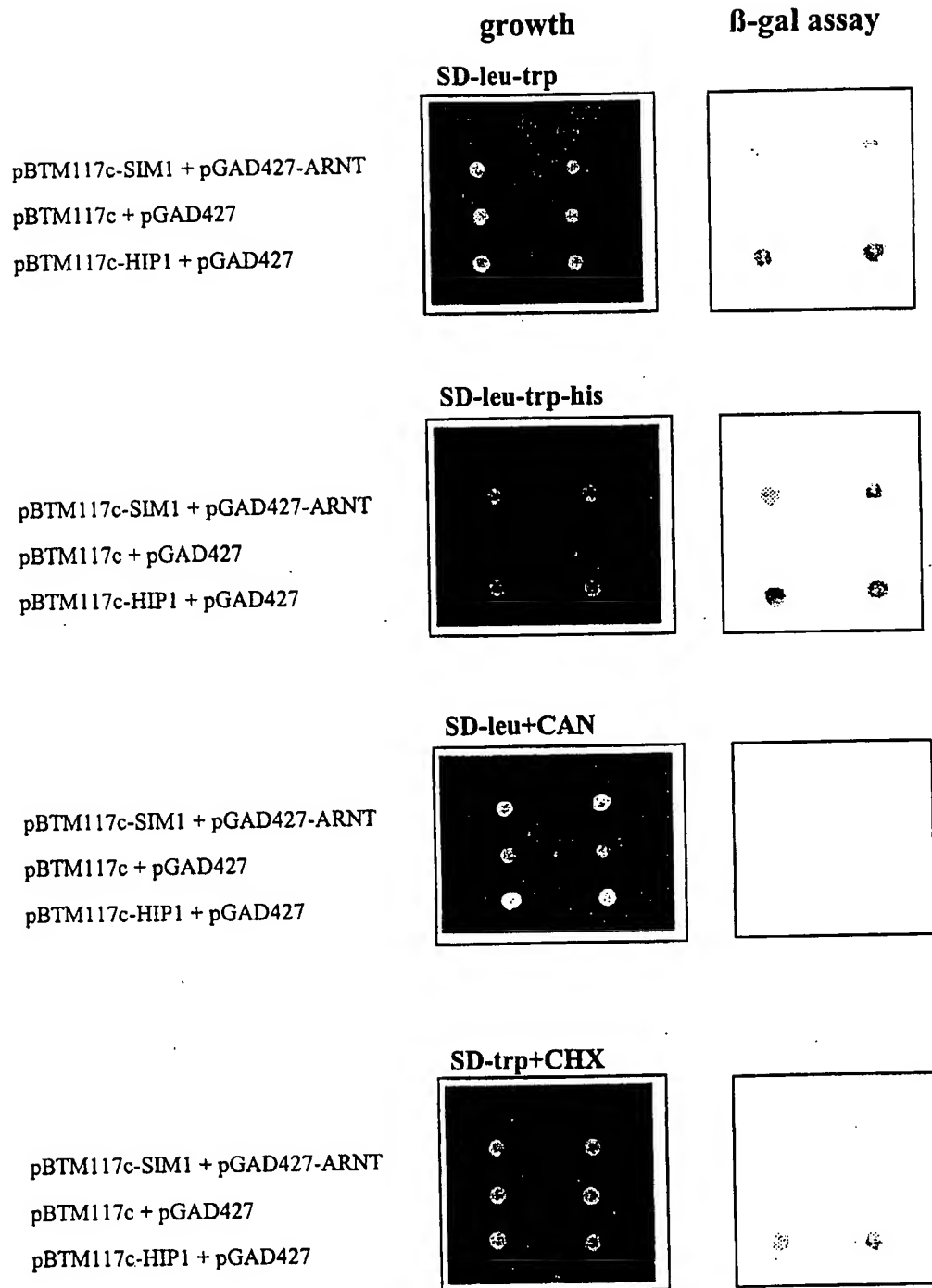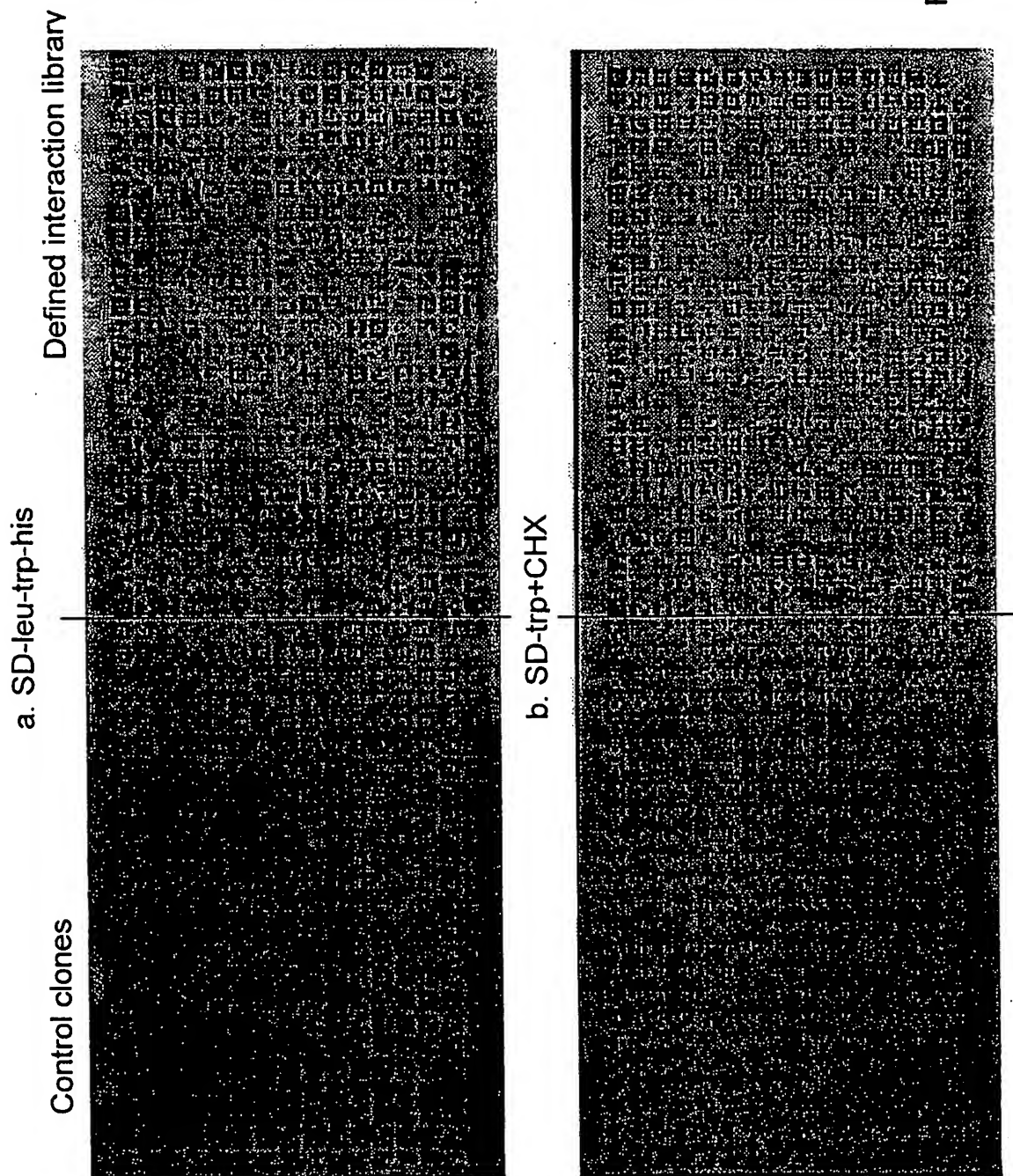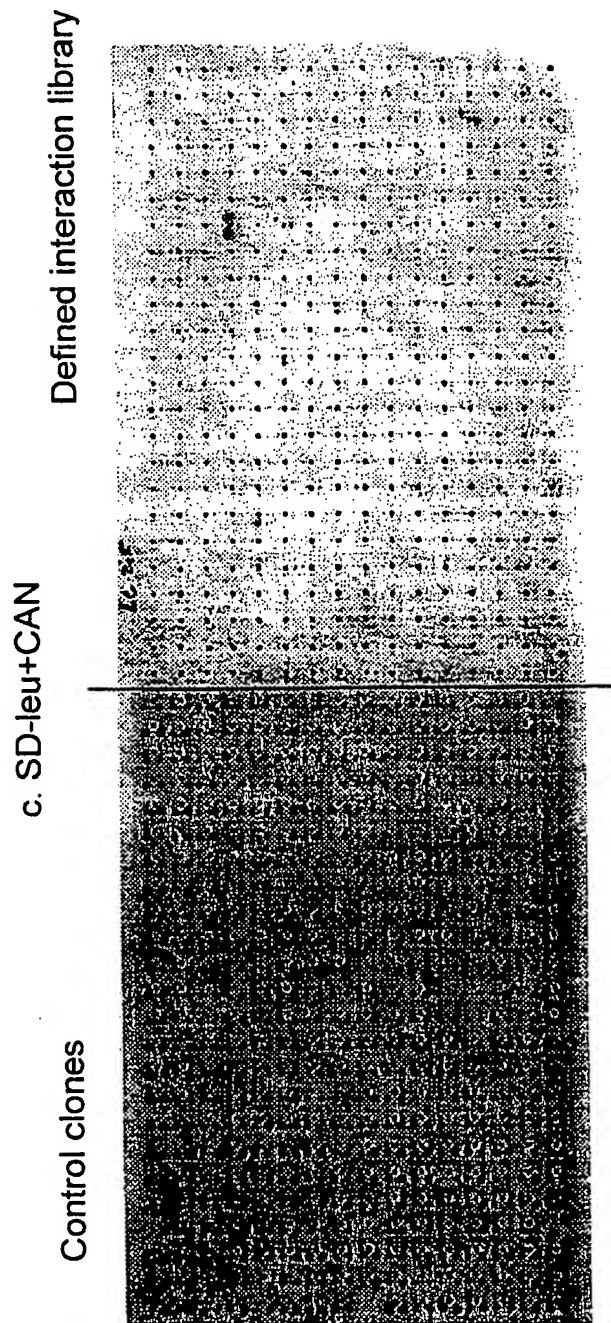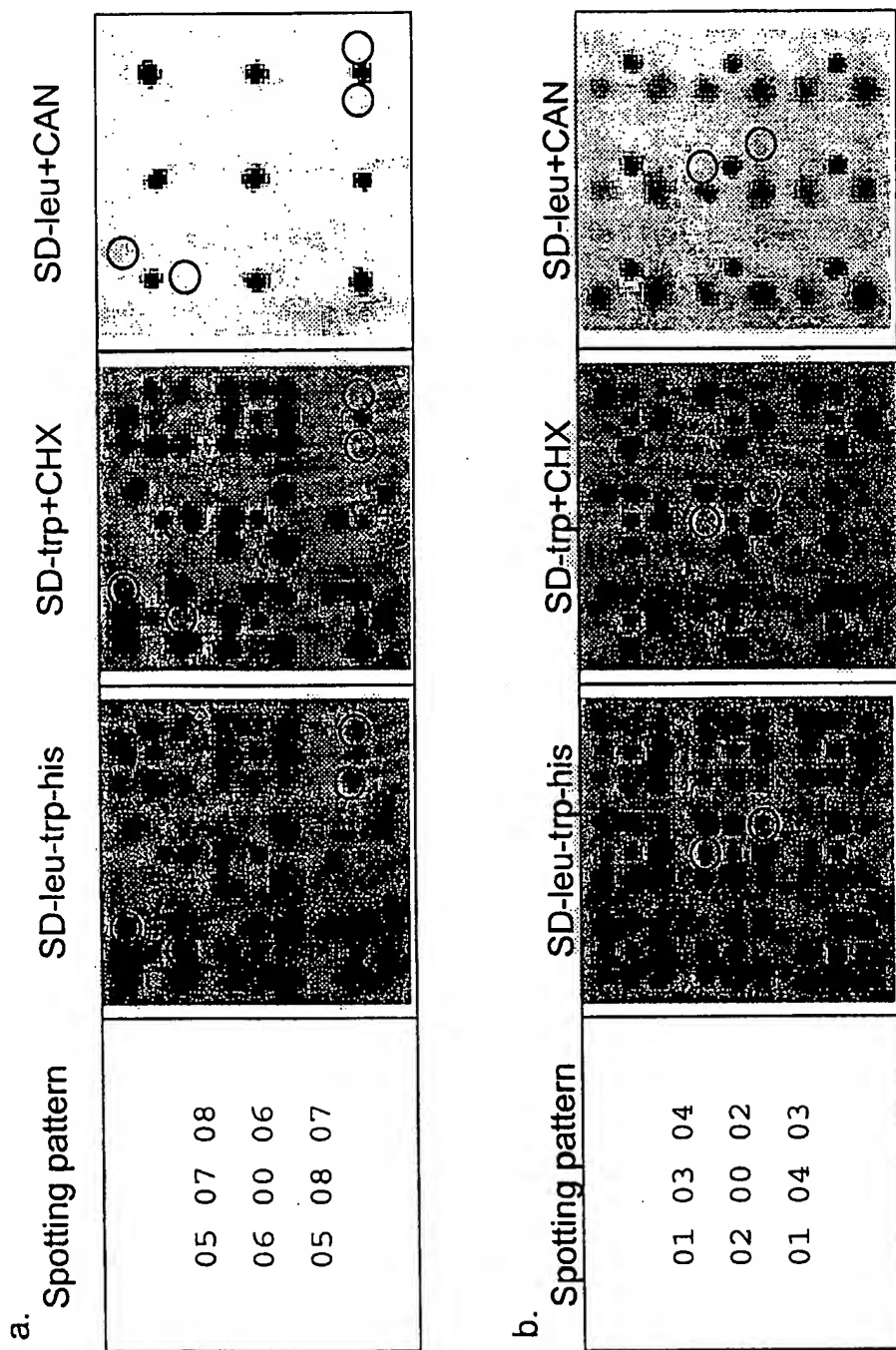
GPC - PositiveFind

	Clone	SD-leu-trp-his	SD-trp+CHX	SD-leu+CAN
675	2O8	2	0	0
687	2O22	2	0	0
689	2P1	3	0	0
690	2P2	2	0	0
693	2P5	3	0	0
696	2P8	3	0	0
699	2P12	3	0	0
709	2P23	2	0	0
710	2P24	2	0	0
711	3A2	3	0	0
712	3A3	3	0	0
713	3A4	3	0	0
714	3A5	3	0	0
716	3A7	3	0	0
717	3A8	3	0	0
718	3A11	3	0	0
719	3A12	2	0	0
720	3A14	3	0	0
721	3A15	3	0	0
722	3A18	2	0	0
723	3A21	2	0	0
724	3A23	2	0	0
725	3A24	3	0	0
727	3B2	3	0	0
728	3B3	3	0	0
729	3B4	3	0	0
730	3B5	3	0	0
731	3B6	3	0	0
732	3B7	3	0	0
733	3B8	3	0	0
734	3B9	3	0	0
735	3B10	3	0	0
736	3B12	3	0	0
737	3B13	3	0	0
738	3B14	3	0	0
739	3B15	3	0	0
741	3B17	2	0	0

b.

GPC - PositiveFind

1	Clone	SD-leu-trp-his	SD-trp+CHX	SD-leu+CAN
1	Clone	SD-leu-trp-his	SD-trp+CHX	SD-leu+CAN
1733	6L22	3	0	0
2302	8N24	2	0	0

Figure 12

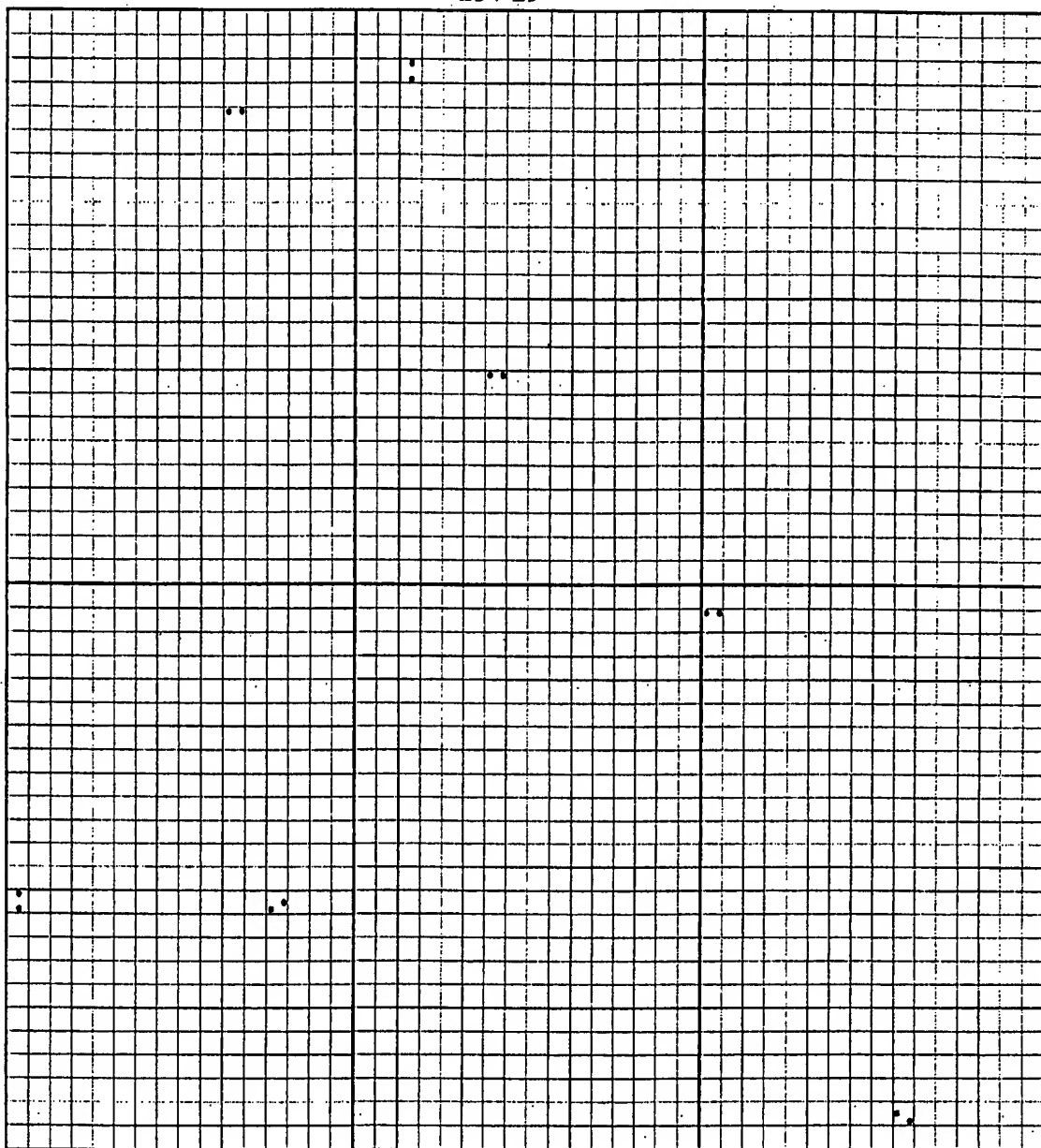SIM1 probe　　　　　　　　ARNT probe
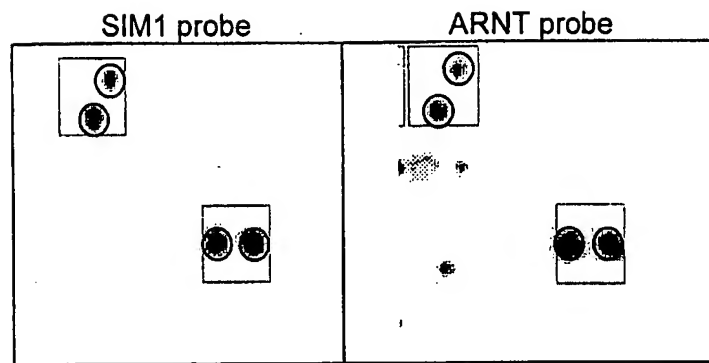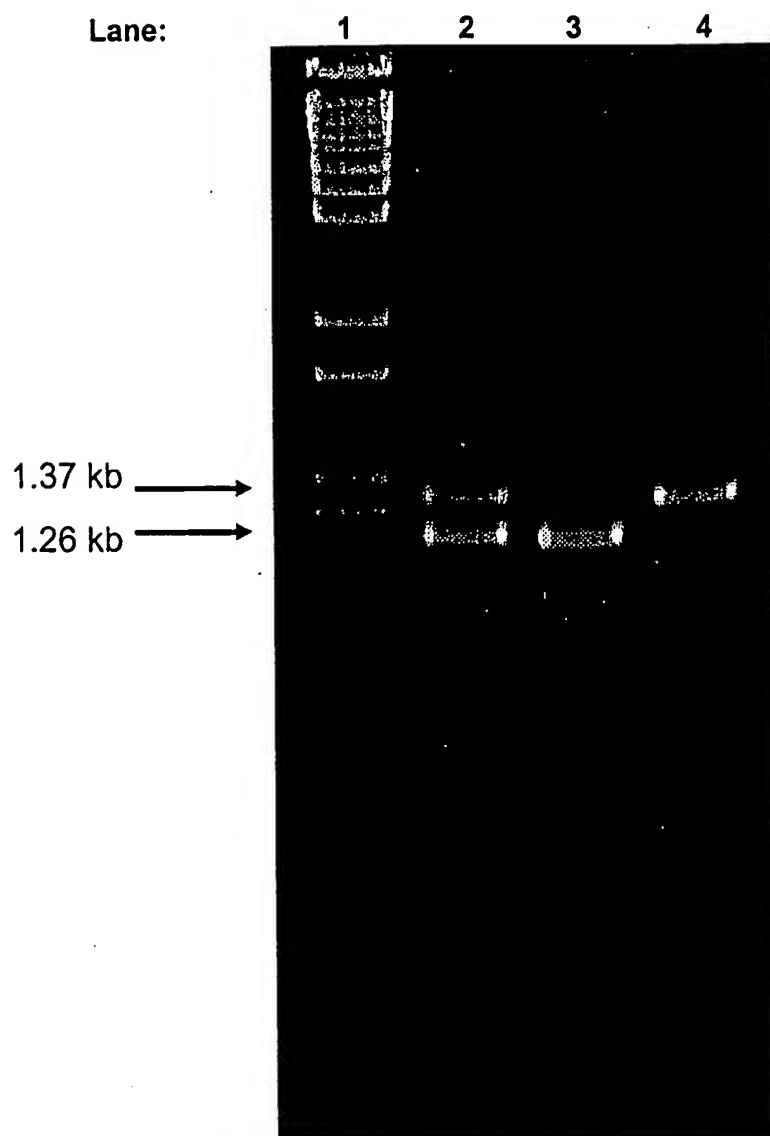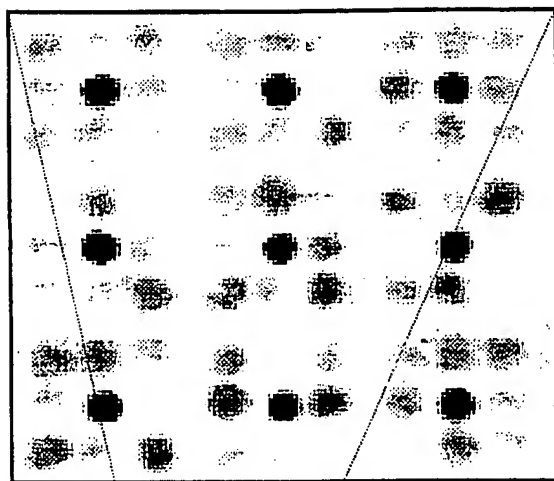
Figure 13

Figure 14

Figure 15
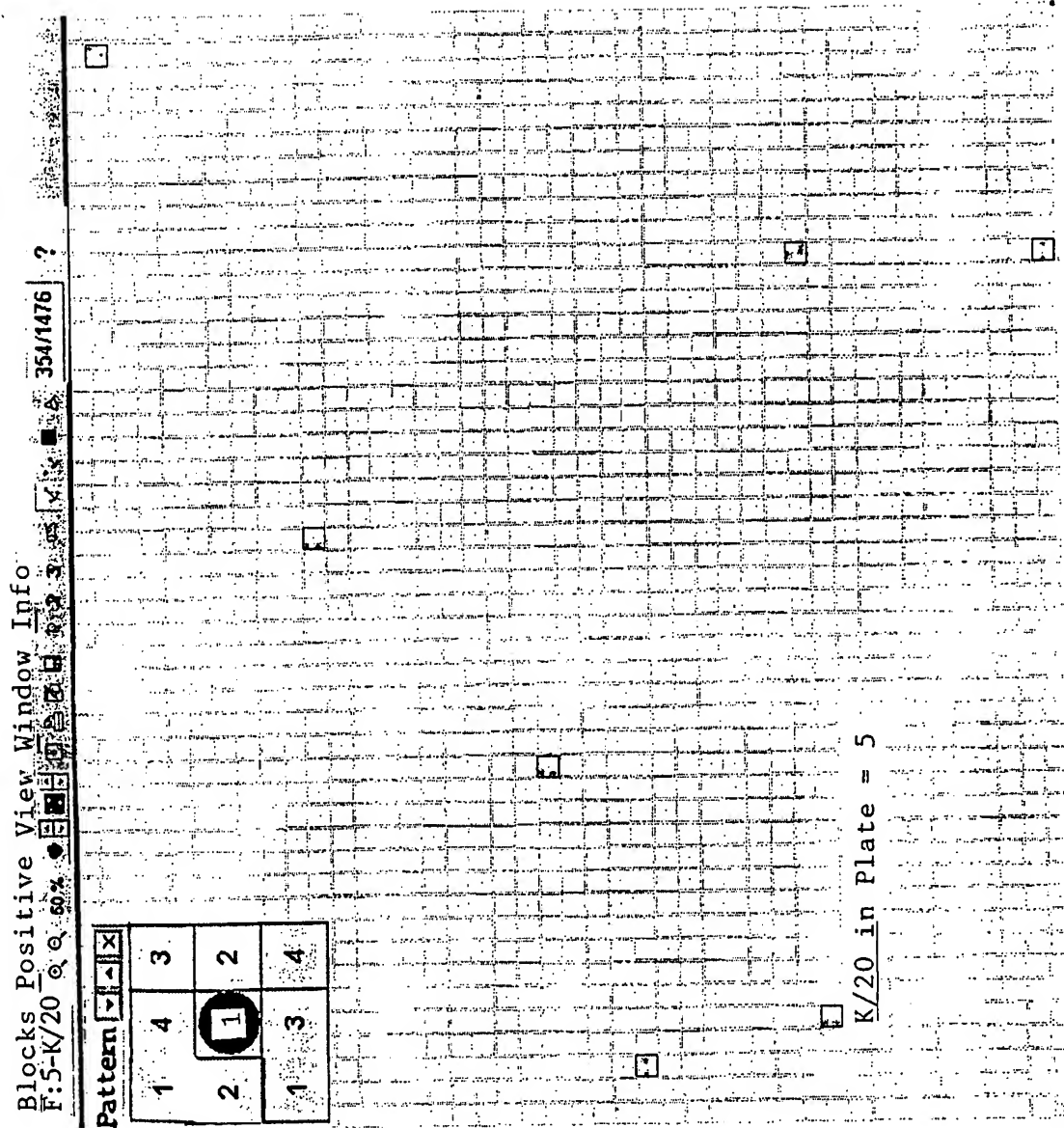
Figure 16

Figure 17
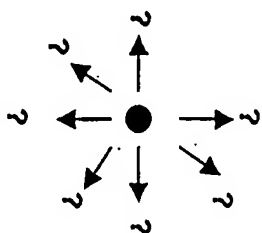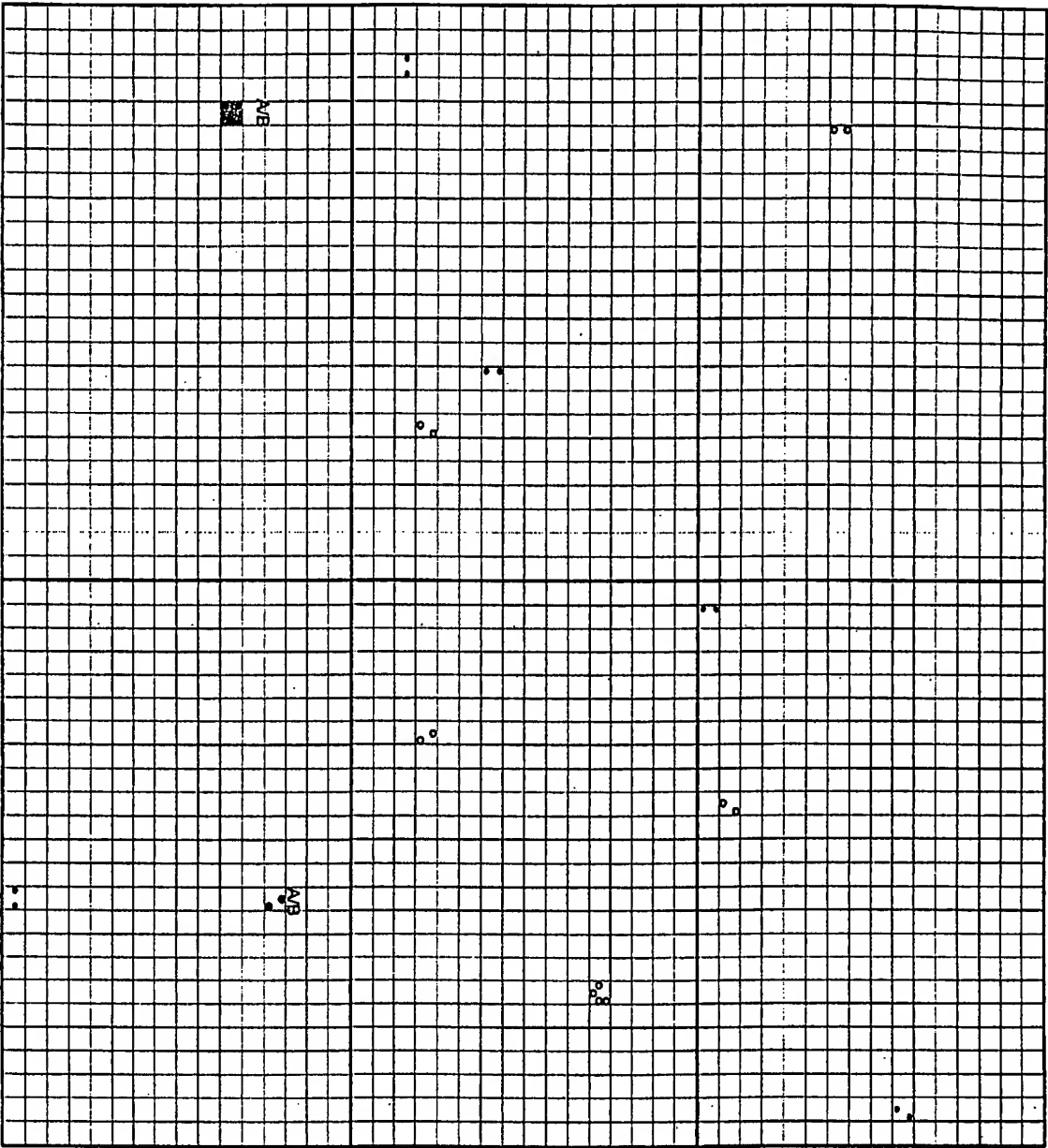
1st Hybridisation

Protein A: ●

Interaction Map

Figure 18

1st Hybridisation

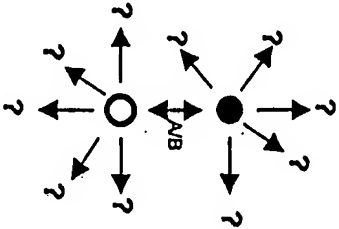Protein A: ●

2nd Hybridisation
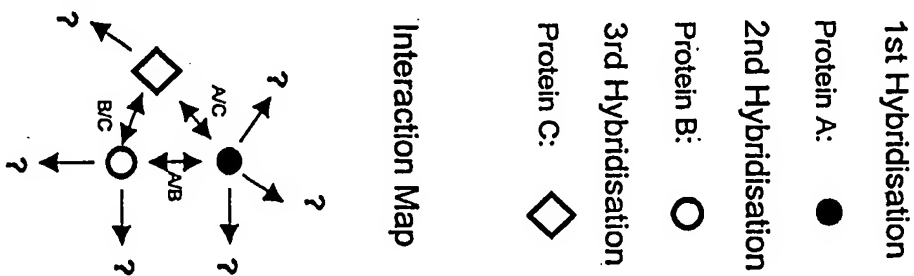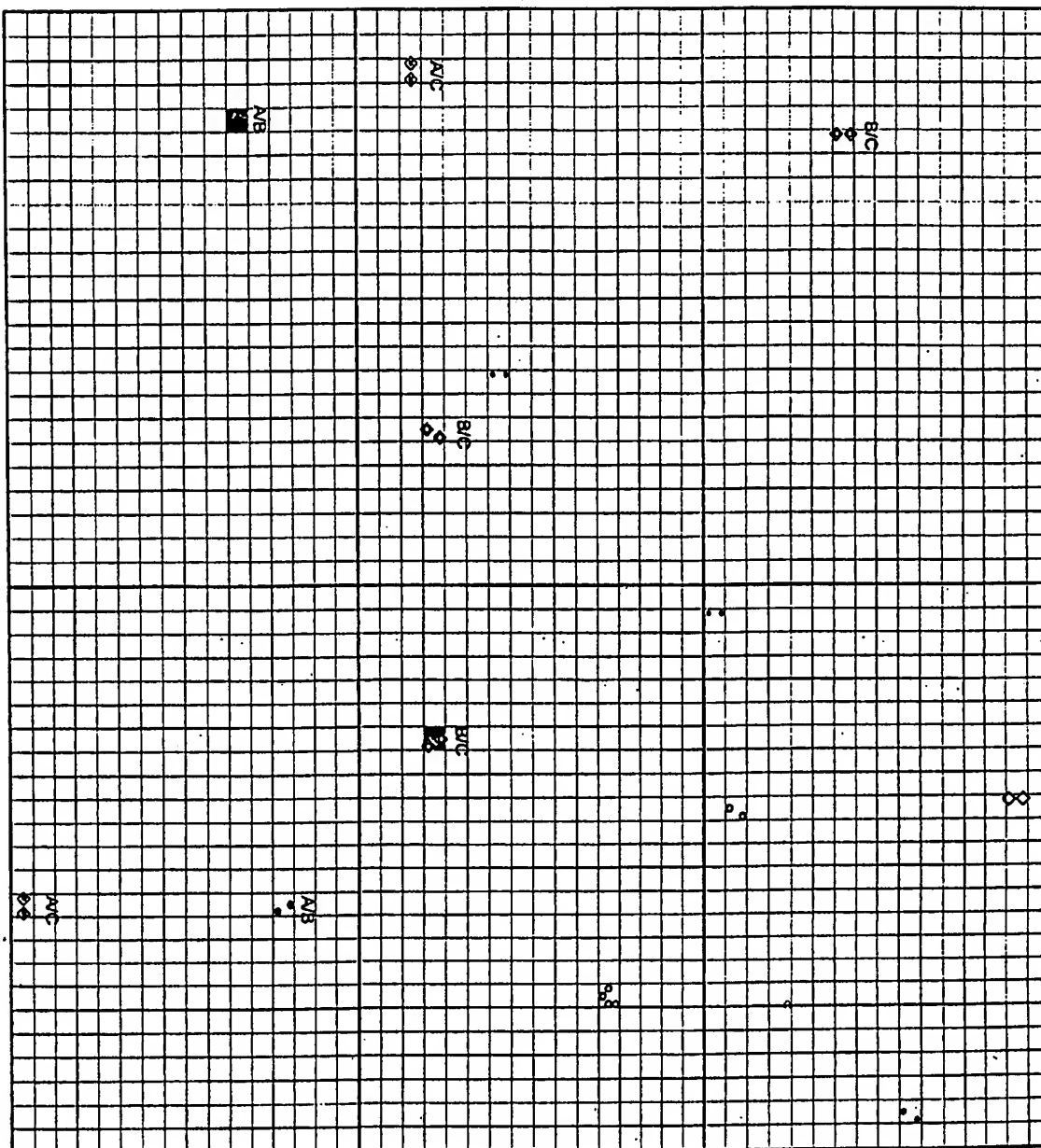
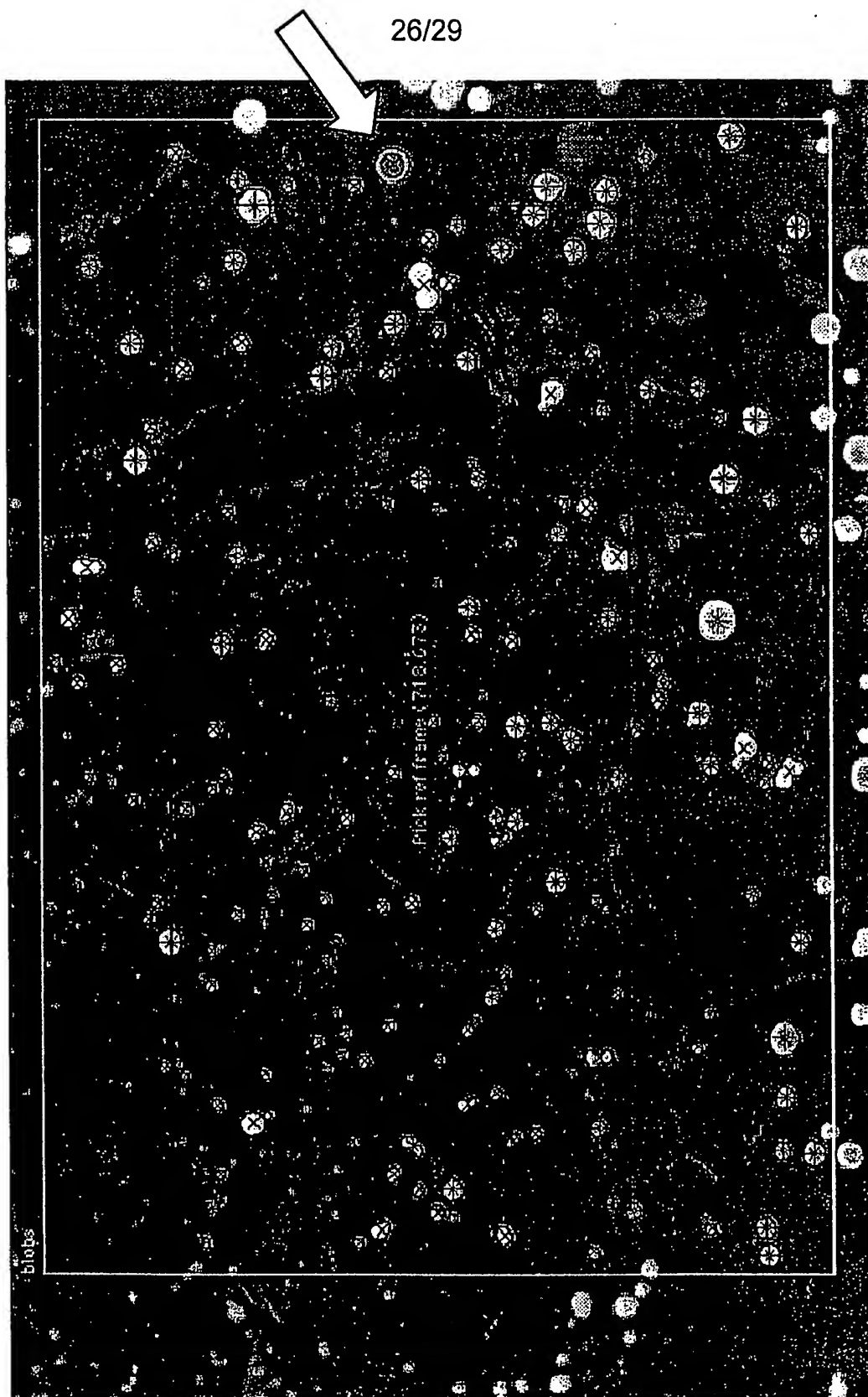Protein B: ○

Interaction Map

Figure 19

26/29

Figure 20

YPD

X-Gal
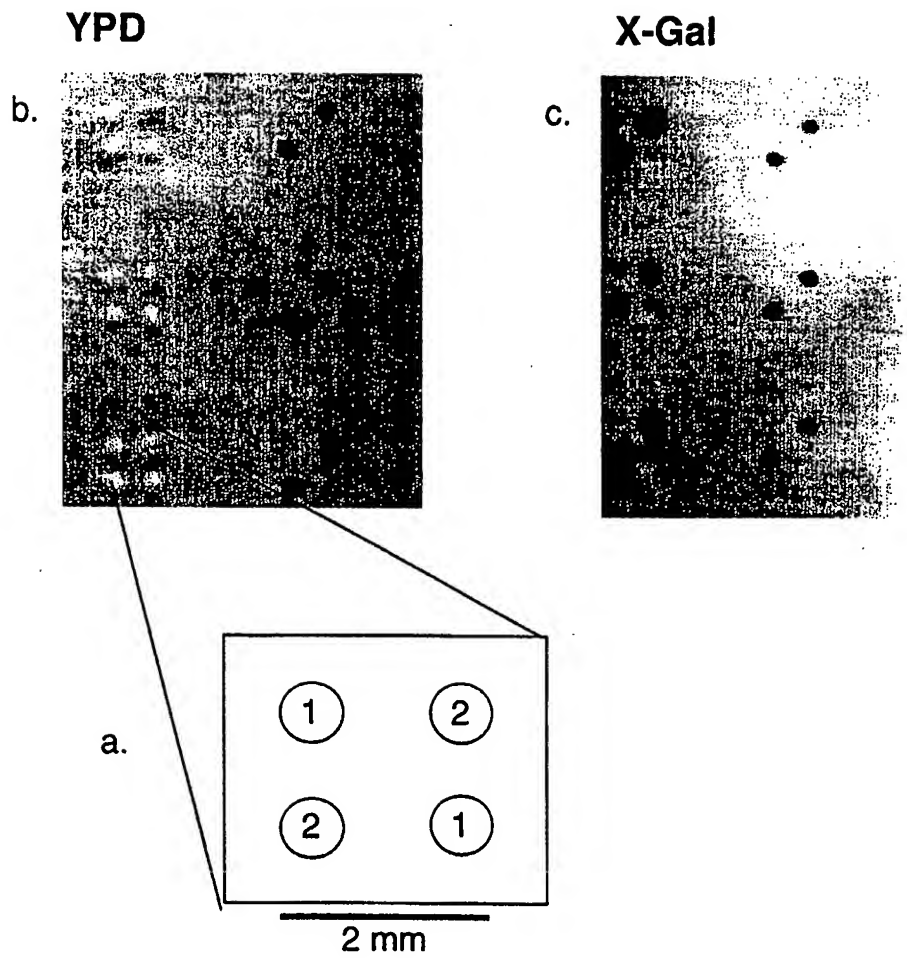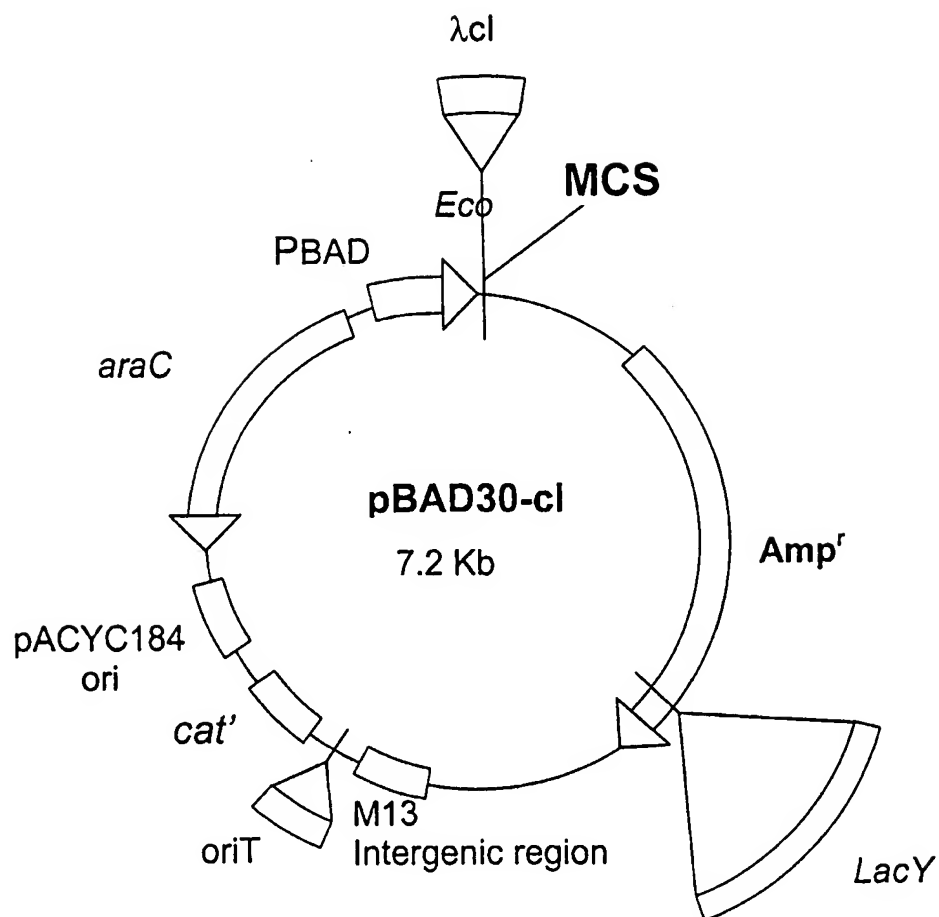
Figure 21
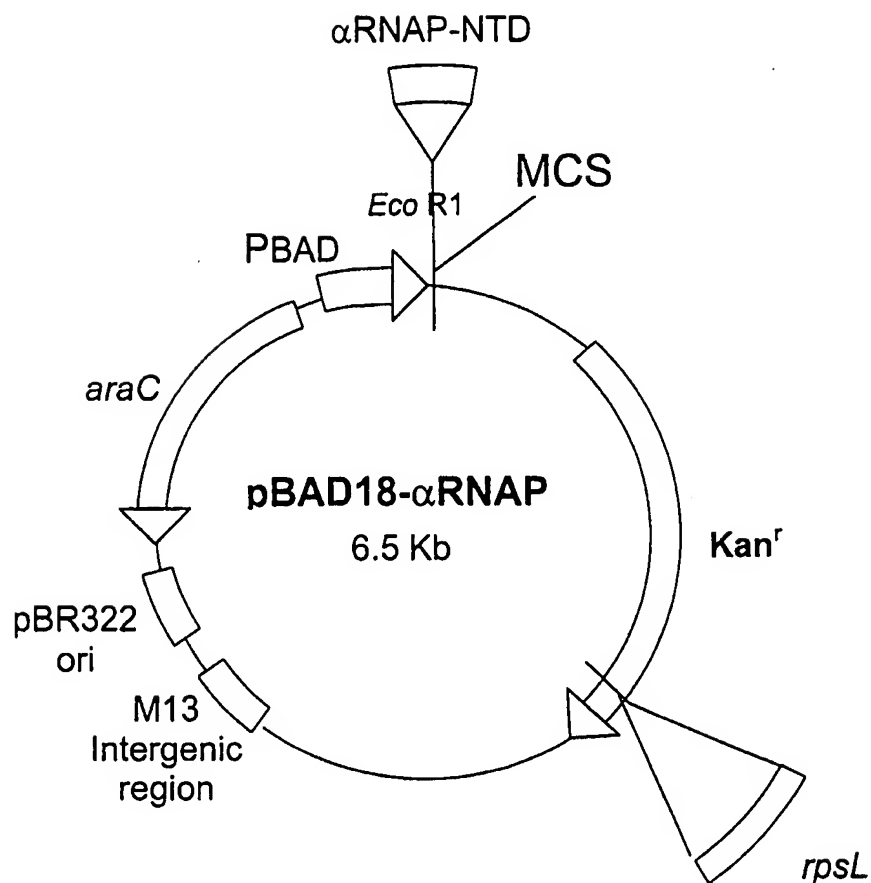
Figure 22 (I)

Figure 22 (II)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/50   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N   C12N   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 32503 A (GEN HOSPITAL CORP ;UNIV JOHNS HOPKINS (US)) 17 October 1996 cited in the application see claim 1	1,4-59, 63-66
Y	see the whole document ---	2
X	EP 0 790 304 A (AMERSHAM INT PLC) 20 August 1997 see column 1 - column 3 see column 11, line 46 - column 12, line 18 --- -/--	1,3-59, 63-66

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 1999	28/04/1999

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Hoekstra, S

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DOVE S L ET AL: "ACTIVATION OF PROKARYOTIC TRANSCRIPTION THROUGH ARBITRARY PROTEIN-PROTEIN CONTACTS" NATURE, vol. 386, 10 April 1997, pages 627-630, XP002050708 cited in the application see the whole document	3
Y	see page 628, right-hand column, last paragraph	2

X,P	WO 97 47763 A (CURAGEN CORP) 18 December 1997 cited in the application see the whole document see example 6.3	2,3

P,A	WO 98 07845 A (JOUNG J KEITH ;DOVE SIMON (US); HARVARD COLLEGE (US); HOCHSCHILD A) 26 February 1998 see claims	1-59, 63-66

3

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:   60-62
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   see FUTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of Invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Claims 60-62 are methods for preparing compositions which are technically limited to molecules identified by the screening methods of claims 1-59. No features of a technical nature are comprised in the claims to either characterise the method steps or these molecules. These claims do hence not comply with all the prescribed requirement of Article 6 and Rule 6.3(a) PCT to such an extend that a meaningful search is not possible for these claims.

This International Searching Authority found multiply (groups of) inventions in this international application, as follows:

1. Claims: 1 entirely and 4-66 partly

   two hybrid screen involving pre-selection in non-yeast hosts

2. Claims: 2 entirely, 4-66 partly
   Two hybrid screen involving pre-selection involving a visual read out system; not host limited.

3. Claims: 3 entirely, 4-66 partly
   Automated two hybrid screen involving pre-selection; not host limited.

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of)
inventions in this international application, as follows:

1. Claims: 1 entirely and 4-66 partly

    Two hybrid screen involving pre-selection in non-yeast hosts

2. Claims: 2 entirely, 4-66 partly

    Two hybrid screen involving pre-selection involving a visual
    read out system; not host limited.

3. Claims: 3 entirely, 4-66 partly

    Automated two hybrid screen involving pre-selection; not
    host limited.

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Claims Nos.:   60-62

Claims 60-62 are methods for preparing compositions which are technically
limited to molecules identified by the screening methods of claims 1-59.
No features of a technical nature are comprised in the claims to either
characterise the method steps or these molecules. These claims do hence
not comply with all the prescribed requirement of Article 6 and Rule
6.3(a) PCT to such an extend that a meaningful search is not possible for
these claims.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9632503	A	17-10-1996	AU	5541296 A	30-10-1996
			CA	2217545 A	17-10-1996
			EP	0830459 A	25-03-1998
			JP	11502717 T	09-03-1999
EP 0790304	A	20-08-1997	AU	1165697 A	17-07-1997
			EP	0868509 A	07-10-1998
			WO	9723609 A	03-07-1997
WO 9747763	A	18-12-1997	AU	3395597 A	07-01-1998
			CA	2257958 A	18-12-1997
WO 9807845	A	26-02-1998	AU	4159697 A	06-03-1998